# The Role of PGC-1α and PGC-1β during Inflammatory Processes in Skeletal Muscle

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von

**Petra S. Eisele**

aus

Deutschland

**Promotionskomitee**

Prof. Dr. Christoph Handschin (Leitung der Dissertation)

Prof. Dr. Thierry Hennet (Vorsitz)

Prof. Dr. Marc Donath

Prof. Dr. Johan Auwerx

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# SUMMARY

Transcription is a fundamental cellular process which is tightly regulated by transcription factors and cofactors to maintain homeostasis, even under changing conditions. The family of peroxisome proliferator-activated receptor (PPAR) γ coactivator (PGC) 1 transcription cofactors comprises PGC-1α, PGC-1β and PRC, which all induce oxidative metabolism including mitochondrial biogenesis and fatty acid oxidation. PGC-1α and PGC-1β have been studied in greater detail than PRC and are predominantly expressed in tissues with high oxidative capacity like brown adipose tissue (BAT), heart and skeletal muscle.

Inflammation is the protective response of the body to infection and injury involving tissue as well as immune cells. During this process, a complex cytokine network governs communication between cells, coordinates elimination of pathogens, regeneration and finally drives resolution of the inflammatory reaction. If persistently active, inflammation however becomes detrimental and in fact, accompanies a large number of chronic diseases like type 2 diabetes.

In skeletal muscle of diabetic patients, levels of PGC-1α and PGC-1β are diminished and a mouse model with skeletal muscle-specific deletion of PGC-1α shows elevated inflammatory markers both locally and systemically. These findings suggest a causal role of PGC-1 coactivators in counteracting inflammation. The work presented here explored the relationship of those coactivators with inflammatory reactions in skeletal muscle *in vitro* and *in vivo*.

I*n vitro*, PGC-1α and PGC-1β were expressed using adenoviral vectors in a C2C12 myotube model. Tumour necrosis factor (TNF) α, different toll-like receptor agonists and free fatty acids all elicited pro-inflammatory cytokine expression that was diminished by both coactivators in a stimulus- and gene-dependent manner. To further elucidate the mechanism behind this repressive action, TNFα treatment was chosen as paradigm. A microarray experiment with subsequent bioinformatic analysis revealed that the NF-κB pathway, which is a major driver of inflammatory reactions, plays an important role in PGC-1-controlled inflammatory gene expression. This prediction was validated as NF-κB reporter gene activity was inhibited by both PGC-1α and PGC-1β. However, neither changes in mRNA nor in protein expression of components of the NF-κB signalling pathway explained the repression. Rather, PGC-1 coactivators affected the post-translational modification of p65, a NF-κB subunit. Diminished phosphorylation at serine 536 precluded complete transcriptional activation by p65 leading to lower cytokine levels.

Protein kinase B (Akt) activity was concomitantly lowered by PGC-1α and PGC-1β. Upon stimulation, Akt initiates IKKα-dependent phosphorylation of p65, which in this case is decreased with lower Akt activity. There are hints that an unknown phosphatase participates in dephosphorylation of p65. Transrepression represents a second link between PGC-1 coactivators and inflammatory reactions: PGC-1α and PGC-1β both induced expression of the nuclear receptor PPARα

which has known anti-inflammatory properties. In fact, inhibition of PPARα in our system recovered pro-inflammatory cytokine levels and thus abolished the repressive effect at least of PGC-1β. Therefore, *in vitro* Akt inhibition and PPARα-mediated transrepression caused by PGC-1 coactivators lead to incomplete activation of p65-dependent transcription of pro-inflammatory cytokines.

Furthermore, PGC-1α but nor PGC-1β induced components of the noncanonical NF-κB signalling pathway while PGC-1β but not PGC-1α decreased expression and DNA binding of the NF-κB subunits p65 and p50 in the basal state. The latter very likely accounts for repression of cytokine expression by PGC-1β without stimulation.

*In vivo*, mouse models with skeletal muscle-specific PGC-1α or PGC-1β overexpression were either injected into *tibialis anterior* with TNFα or lipopolysaccharide or exposed to a single bout of downhill running on a treadmill. Pro-inflammatory cytokine levels neither differed systemically, nor locally in skeletal muscle. Unexpectedly, transgenic animals displayed higher numbers of macrophages in this tissue, which were however skewed towards an alternatively activated (M2) phenotype. The presence of M2 signature cytokines further supported the influence of muscle PGC-1 expression on alternative macrophage activation creating an anti-inflammatory environment. Most of these cytokines however derived from immune cells as only a PGC-1-mediated suppression of IL-12 expression could be replicated *in vitro*. Thus, insufficient IL-12 levels are presumably the root of M2 skewing.

The study presented here establishes PGC-1α and PGC-1β as regulators of immune function in skeletal muscle. A direct effect on NF-κB signalling and pro-inflammatory cytokine expression was detected *in vitro* while *in vivo*, those coactivators more indirectly influenced the inflammatory milieu through alternative macrophage activation. In the context of metabolic disorders like type 2 diabetes, these findings are of interest because low PGC-1 expression and chronic inflammation accompany diseases progression. It appears that targeting PGC-1α and PGC-1β in skeletal muscle offers a potential treatment strategy to avoid entering the vicious circle of deteriorating metabolic function and heightened inflammation. The results further provide evidence that metabolic and inflammatory pathways are more tightly linked than previously reported and skeletal muscle should be considered an organ as much involved into metabolic fluxes as in immune responses contributing to an overall healthy state.

# ZUSAMMENFASSUNG

Transkription ist ein grundlegender, zellulärer Prozess, der durch Transkriptionsfaktoren und Kofaktoren genauestens geregelt wird, um Homöostase auch unter sich ändernden Bedingungen zu erhalten. Zur Familie der „peroxisome proliferator-activated receptor (PPAR) γ coactivator (PGC) 1"-Transkriptionskofaktoren gehören PGC-1α, PGC-1β und PRC, die alle den oxidativen Stoffwechsel anregen, was mitochondrielle Biogenese und Fettsäureoxidation einschliesst. PGC-1α und PGC-1β wurden bereits genauer untersucht als PRC und sind hauptsächlich in Geweben mit hoher oxidativer Kapazität wie braunem Fettgewebe, Herz und Skelettmuskel vorhanden.

Entzündung ist die schützende Antwort des Körpers auf Infektionen und Verletzungen, an der sowohl Körper- als auch Immunzellen beteiligt sind. Während dieses Prozesses stimmt ein komplexes Netzwerk aus Zytokinen die Kommunikation zwischen diesen Zellen ab, lenkt die Beseitigung von Krankheitserregern, die Heilung und veranlasst schliesslich die Auflösung der entzündlichen Reaktion. Wenn Entzündungen jedoch chronisch werden, können sie schädliche Wirkungen entfalten, und tatsächlich treten sie bei vielen chronischen Erkrankungen wie z.B. Typ-2-Diabetes als Begleiterscheinung auf.

Diabetiker weisen im Skelettmuskel einen reduzierten Gehalt an PGC-1α und PGC-1β auf, und in einem Mausmodell, dem PGC-1α im Skelettmuskel fehlt, werden sowohl im Blutkreislauf als auch lokal erhöhte Entzündungswerte gemessen. Diese Ergebnisse deuten darauf hin, dass PGC-1 Koaktivatoren eine ursächliche Rolle bei der Bekämpfung von Entzündungen spielen könnten. Die vorliegende Arbeit setzt sich mit der Beziehung zwischen diesen Koaktivatoren und Entzündungsreaktionen im Skelettmuskel *in vitro* und *in vivo* auseinander.

I*n vitro* wurden PGC-1α und PGC-1β von adenoviralen Vektoren in einem C2C12 Myotubenmodell exprimiert. Tumornekrosefaktor (TNF) α, spezifische Aktivatoren verschiedener toll-like Rezeptoren und freie Fettsäuren riefen alle die Expression proentzündlicher Zytokine hervor, die von beiden Koaktivatoren in einer stimulus- und genabhängigen Art und Weise beeinträchtigt wurde. Um den Mechanismus dieser Repression genauer zu verstehen, wurde die Behandlung mit TNFα als Musterbeispiel gewählt. Ein Microarray-Experiment und dessen anschliessende, bioinformatische Auswertung ergaben, dass der NF-κB Signalweg, einer der Haupteinflussfaktoren entzündlicher Reaktionen, eine entscheidende Rolle bei der von PGC-1 kontrollierte Genexpression spielt. Diese Vorhersage wurde dadurch bestätigt, dass PGC-1α und PGC-1β beide die Aktivität eines NF-κB Reportergens einschränkten. Allerdings konnte diese Repression weder durch Änderungen des mRNA-Gehalts noch des Proteingehalts einzelner Komponenten des NF-κB Signalweges erklärt werden. Die PGC-1 Koaktivatoren beeinflussten vielmehr die posttranslationale Modifikation von p65, einer NF-κB Untereinheit. Die verringerte Phosphorylierung am Serin 536 verhinderte die vollständige transkriptionelle Aktivität von p65, was zu niedrigeren Zytokinspiegeln führte.

Parallel dazu war die Aktivität der Proteinkinase B (Akt) durch PGC-1α und PGC-1β reduziert. Akt ist für die IKKα-abhängige Phosphorylierung von p65 nach einem Stimulus verantwortlich, die in diesem Falle durch die geringere Akt Aktivität vermindert war. Es gibt ausserdem Hinweise, dass eine unbekannte Phosphatase an der Dephosphorylierung von p65 beteiligt ist. Ein zweites Bindeglied zwischen PGC-1 Koaktivatoren und Entzündungsreaktionen ist die Transrepression: PGC-1α und PGC-1β erhöhten beide die Expression des nukleären Rezeptors PPARα, der antientzündliche Eigenschaften besitzt. Demzufolge normalisierte die Hemmung von PPARα tatsächlich die Zytokinspiegel in unserem System und hob damit die repressive Wirkung zumindest von PGC-1β auf. Somit führen Akt Hemmung und PPARα-vermittelte Transrepression *in vitro* zu unvollständiger Aktivierung p65-abhängiger proentzündlicher Gentranskription.

Des Weiteren steigerte PGC-1α, aber nicht PGC-1β, die Expression von Komponenten des nicht-kanonischen NF-κB Signalweges, wohingegen PGC-1β, aber nicht PGC-1α, sowohl den Gehalt als auch die DNS-Bindung der NF-κB Untereinheiten p65 und p50 im Grundzustand verminderte. Letzteres erklärt sehr wahrscheinlich die Repression der Zytokinexpression durch PGC-1β in Abwesenheit eines Stimulus.

*In vivo* wurde Mausmodellen mit skelettmuskelspezifischer PGC-1α oder PGC-1β Überexpression entweder TNFα oder Lipopolysaccharid in den *Musculus tibialis anterior* gespritzt oder sie mussten einmalig bergab auf einem Laufband laufen. Der Spiegel proentzündlicher Zytokine unterschied sich weder systemisch noch lokal im Skelettmuskel. Die transgenen Tiere hatten aber unerwartet eine grössere Anzahl Makrophagen im Gewebe, die sich jedoch stärker in einem alternativen Aktivierungszustand (M2) befanden. Der Nachweis von Zytokinen, die für diesen M2 Zustand charakteristisch sind, deutete ebenfalls auf den Einfluss von Muskel-PGC-1 auf die alternative Makrophagenaktivierung hin, was zur Schaffung eines antientzündlichen Milieus beitrug. Der Grossteil dieser Zytokine stammte jedoch von Immunzellen, da nur die PGC-1-bedingte Hemmung der IL-12 Expression *in vitro* repliziert werden konnte. Demzufolge sind zu niedrige IL-12-Spiegel wahrscheinlich der Grund für die Verschiebung des M1-M2 Gleichgewichts.

Die hier vorgestellte Studie etabliert PGC-1α und PGC-1β als Regulatoren der Immunfunktion im Skelettmuskel. Ein direkter Effekt auf den NF-κB Signalweg und auf proentzündliche Zytokinexpression konnte *in vitro* nachgewiesen werden, wohingegen diese Koaktivatoren *in vivo* vor allem indirekt auf das Entzündungsumfeld durch alternative Makrophagenaktivierung wirkten. Im Zusammenhang mit Stoffwechselkrankheiten wie z.B. Typ-2-Diabetes sind diese Ergebnisse von Interesse, da niedrige PGC-1 Expression und eine chronische Entzündung den Krankheitsverlauf begleiten. Behandlungsmethoden, die auf PGC-1α und PGC-1β im Skelettmuskel abzielen, könnten so möglicherweise helfen, den Teufelskreis aus sich verschlechterndem, metabolischem Zustand und gesteigerter Entzündung zu durchbrechen. Die Ergebnisse weisen ausserdem darauf hin, dass Stoffwechsel- und Entzündungssignalwege enger miteinander verbunden sind als bisher angenommen, und dass der Skelettmuskel nicht nur ein

Stoffwechselorgan ist, sondern auch an Immunantworten beteiligt, und dadurch zu einem gesunden Gesamtzustand beiträgt.

# ABBREVIATIONS

| Abbreviation | Full name |
|---|---|
| AD | activation domain |
| ALAS-1 | 5-aminolevulinate synthase |
| ALS | amyotrophic lateral sclerosis |
| AMPK | adenosine monophosphate-activated protein kinase |
| AP-1 | activator protein 1 |
| ATF2 | activating transcription factor 2 |
| ATM | adipose tissue macrophage |
| BAF60 | BRG-1-associated factor 60 |
| BAFFR | B-cell activating factor receptor |
| BAT | brown adipose tissue |
| Bcl3 | B-cell lymphoma 3 |
| Bcl6 | B-cell lymphoma 6 |
| BMI | body mass index |
| CaMKIV | calcium/calmodulin-dependent protein kinase type IV |
| cAMP | cyclic adenosine monophosphate |
| CAR | constitutive androstane receptor |
| CBP | CREB-binding protein |
| CCL1 | C-C motif chemokine 1 |
| CCL17 | C-C motif chemokine 17 |
| CCL2 | C-C motif chemokine 2 (MCP-1) |
| CCL22 | C-C motif chemokine 22 |
| CCL3 | C-C motif chemokine 3 (MIP-1α) |
| CCL5 | C-C motif chemokine 5 (RANTES) |
| ChIP | chromatin immunoprecipitation |
| CKII | casein kinase II |
| CMV | cytomegalovirus |
| CnA | calcineurin A |
| COPD | chronic obstructive pulmonary disease |
| COX IV | cytochrome c oxidase subunit 4 |
| COX2 | cyclooxygenase 2 |
| CPT1 | carnitine O-palmitoyltransferase 1 |
| CRE | cyclic adenosine monophosphate response element |
| CREB | cyclic adenosine monophosphate response element-binding protein |
| CRP | C-reactive protein |
| CtBP | C-terminal binding protein |
| Ctrl | control |
| CXCL1 | C-X-C motif chemokine 1 |
| CXCL10 | C-X-C motif chemokine 10 |
| CYP7A1 | cholesterol 7-α-hydroxylase 1 |
| DAPI | 4',6-diamidino-2-phenylindole |
| DAX-1 | dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on X chromosome, gene 1 |

| | |
|---|---|
| DD | death domain |
| DMD | Duchenne muscular dystrophy |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | deoxyribonucleic acid |
| DNMT3B | DNA methyltransferase 3B |
| DRIP | vitamin D receptor-interacting protein |
| ELISA | enzyme-linked immunosorbent assay |
| ER | endoplasmic reticulum |
| ERα | estrogen receptor α |
| ERK | extracellular signal-related kinase |
| ERRα | estrogen-related receptor α |
| ERRγ | estrogen-related receptor γ |
| ETC | electron transport chain |
| FADD | Fas–associated DD |
| FFA | free fatty acid |
| FGF21 | fibroblast growth factor 21 |
| FoxO1 | forkhead box protein O1 |
| FoxO3a | forkhead box protein O3a |
| Fsk | forskolin |
| FXR | farnesoid X receptor |
| G-6-Pase | glucose-6-phosphatase |
| GCN5 | general control of amino acid synthesis 5 |
| GFP | green fluorescent protein |
| GLUT4 | glucose transporter 4 |
| Gpx-1 | glutathione peroxidise 1 |
| GR | glucocorticoid receptor |
| GRIP1 | glutamate receptor-interacting protein 1 |
| GSK3β | glycogen synthase kinase 3β |
| h | hour |
| H&E | Mayer's hematoxylin and eosin |
| HAT | histone acetyltransferase |
| HD | Huntington´s disease |
| HDAC | histone deacetylase |
| HFD | high fat diet |
| HIF1α | hypoxia-inducible factor 1α |
| HNF4α | hepatic nuclear factor 4α |
| HO-1 | heme oxygenase 1 |
| Hsp27 | heat shock protein 27 |
| *i.m.* | interamuscular |
| *i.p.* | intraperitoneal |
| IFNγ | interferon γ |
| IgG | immunoglobulin G |
| IκB | inhibitor of NF-κB |
| IKK | IκB kinase |
| IL-10 | interleukin 10 |
| IL-12 | interleukin 12 |
| IL-13 | interleukin 13 |

| | |
|---|---|
| IL-15Rα | interleukin 15 receptor α |
| IL-1β | interleukin 1β |
| IL-1Ra | interleukin 1 receptor antagonist |
| IL-4 | interleukin 4 |
| IL-6 | interleukin 6 |
| IL-6R | interleukin 6 receptor |
| IRAK1 | interleukin 1 receptor-asscoiated kinase 1 |
| IRAK4 | interleukin 1 receptor-asscoiated kinase 4 |
| IRE | insulin response element |
| IRF3 | interferon regulatory factor 3 |
| IRF7 | interferon regulatory factor 7 |
| Irs1 | insulin receptor substrate 1 |
| JAK | Janus kinase |
| JNK | c-Jun N-terminal kinase |
| kb | kilobase |
| KLF4 | Krüppel-like factor 4 |
| LPS | lipopolysaccharide |
| LTβR | lymphotoxin β receptor |
| LXR | liver X receptor |
| Ly6C | lymphocyte antigen 6C |
| Mal | Myd88 adaptor-like protein |
| MAPK | mitogen-activated protein kinase |
| MCAD | medium-chain acyl-coenzyme A dehydrogenase |
| MCK | muscle creatine kinase |
| MCKα | PGC-1α skeletal muscle-transgenic mice |
| MCKβ | PGC-1β skeletal muscle-transgenic mice |
| MCP-1 | monocyte chemoattractant protein 1 (CCL2) |
| MCSF | macrophage colony-stimulating factor |
| MEF2 | myocyte-specific enhancer factor 2 |
| MEK1/2 | MAPK/ERK kinase1/2 |
| min | minute |
| MIP-1α | macrophage inflammatory protein 1α (CCL3) |
| MK2 | MSK kinase 2 |
| MKO | Mice with skeletal muscle-specific deletion of PGC-1α |
| MRC1 | macrophage mannose receptor 1 (CD206) |
| mRNA | messenger ribonucleic acid |
| Ms1 | manuscript 1 |
| Ms2 | manuscript 2 |
| MSK1 | mitogen- and stress-activated protein kinase 1 |
| MSR-1 | macrophage scavenger receptor 1 (CD204) |
| MuRF1 | muscle-specific RING finger protein 1 |
| Myd88 | myeloid differentiation primary response gene 88 |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NcoR | nuclear receptor corepressor 1 |
| NEMO | NF-κB essential modulator |
| NF-κB | nuclear factor κ-light-chain-enhancer of activated B cells |
| NFAT | nuclear factor of activated T cells |

| | |
|---|---|
| NIK | NF-κB inducing kinase |
| NO | nitric oxide |
| Nox-1 | NADPH oxidase 1 |
| NR | nuclear receptor |
| NRF1 | nuclear respiratory factor 1 |
| NRF2 | nuclear respiratory factor 2 |
| nTrip6 | nuclear TR-interacting protein 6 |
| Nurr1 | NUR-related factor 1 |
| OA | okadaic acid |
| p160 mbp | p160 myb binding protein |
| PAMP | pathogen-associated molecular pattern |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PDC | pyruvate dehydrogenase complex |
| PDK4 | pyruvate dehydrogenase kinase 4 |
| PEPCK | phosphoenolpyruvate carboxykinase |
| PGC-1α | peroxisome proliferator-activated receptor γ coactivator 1α |
| PGC-1β | peroxisome proliferator-activated receptor γ coactivator 1β |
| PI3K | phosphatidylinositol 4,5-bisphosphate 3-kinase |
| PKA | protein kinase A |
| PKB/Akt | protein kinase B/Akt |
| PKC | protein kinase C |
| PP1 | protein phosphatase 1 |
| PP2A | protein phosphatase 2A |
| PPARα | peroxisome proliferator-activated receptor α |
| PPARγ | peroxisome proliferator-activated receptor γ |
| PRC | PGC-1-related coactivator |
| PRDM16 | PR domain zinc finger protein 16 |
| PRMT1 | protein arginine methylatransferase 1 |
| PTM | post-translational modification |
| PTX3 | pentaxin-related protein 3 |
| PXR | pregnane X receptor |
| RANK | receptor activator of NF-κB |
| RHD | Rel homology domain |
| RIP1 | receptor-interacting protein 1 |
| ROS | reactive oxygen species |
| RSK1 | ribosomal S6 kinase 1 |
| RT-PCR | real-time polymerase chain reaction |
| RXR | retinoid X receptor |
| $SCF^{Cdc4}$ | Skp1/Cullin/F-box cell division control 4 |
| sed | sedentary |
| SEM | standard error of the mean |
| SeP | selenoprotein P |
| SHP | small heterodimer partner |
| siRNA | small interfering RNA |
| Sirt1 | sirtuin 1 |

| | |
|---|---|
| SMILE | SHP interacting leucine zipper protein |
| SMRT | silencing mediator of retinoic acid and thyroid hormone receptor |
| SOCS1 | suppressor of cytokine signalling 1 |
| SOCS3 | suppressor of cytokine signalling 3 |
| Sod2 | superoxide dismutase [Mn], mitochondrial |
| SRC-1 | steroid receptor 1 |
| SREBP1a | sterol regulatory element-binding protein 1a |
| STAT1 | signal transducer and activator of transcription 1 |
| STAT3 | signal transducer and activator of transcription 3 |
| STAT6 | signal transducer and activator of transcription 6 |
| SWI/SNF | switch/sucrose nonfermentable complex |
| TA | tibialis anterior muscle |
| TAD | transactivation domain |
| TAK1 | transforming growth factor β activating kinase 1 |
| TANK | TRAF family member-associated NF-κB activator |
| TBK1 | TANK-binding kinase 1 |
| TBL-1 | transducin β-like protein 1X |
| TBLR1 | TBL-1-related protein 1 |
| TBP | TATA-Box binding protein |
| TCA | tricarboxylic acid |
| TF | transcription factor |
| Tfam | transcription factor A, mitochondrial |
| TGFβ | transforming growth factor β |
| Th1 | T helper cell 1 |
| Th2 | T helper cell 2 |
| TIR | toll interleukin 1 receptor domain |
| TLR | toll-like receptor |
| TNFα | tumor necrosis factor α |
| TNFR1 | tumor necrosis factor receptor 1 |
| TNFR2 | tumor necrosis factor receptor 2 |
| TR | thyroid hormone receptor |
| TRADD | TNFR-associates death domain protein |
| TRAF2 | TNFR-associated factor 2 |
| TRAF3 | TNFR-associated factor 3 |
| TRAF6 | TNFR-associated factor 6 |
| TRAM | TRIF-related adaptor molecule |
| TRAP | thyroid receptor-associated protein |
| Treg | regulatory T cell |
| TRIF | TIR domain-containing adaptor molecule |
| VCAM1 | vascular cell adhesion molecule 1 |
| VDR | vitamin D receptor |
| VEGF | vascular endothelial growth factor |
| WAT | white adipose tissue |
| WIP-1 | wt-p53-induced phosphatase |
| WT | wild-type |
| YY1 | yin and yang 1 |

# 1 INTRODUCTION

## 1.1 The PGC-1 family of transcription coactivators

### 1.1.1 Structure and molecular mechanism of action

Transcription is a fundamental, biological process that requires complex regulation. In a first step of many ultimately leading to gene expression, transcription factors bind to specific DNA sequences, so-called response elements, in the promoters of target genes. Subsequent recruitment of chromatin remodelling enzymes and the RNA polymerase complex then enables initiation of transcription. Transcription coregulators confer specificity and either potentiate (coactivators) or constrain (corepressors) initiation through interaction with specific transcription factors. One such example is the peroxisome proliferator-activated receptor (PPAR) γ coactivator 1 (PGC-1) family of transcription coactivators which comprises 3 members: PGC-1α, PGC-1β and PGC-1-related coactivator (PRC). They all share a similar structure with an N-terminal activation domain (AD), one or several LXXLL-motifs (where L is leucine and X is any amino acid) and a C-terminal RNA recognition and splicing domain (Fig. 1A). PGC-1α and PGC-1β further comprise an inhibitory region of about 200 amino acids adjacent to the AD. The main mechanism by which PGC-1s facilitate transcription after binding to a transcription factor is the simultaneous recruitment of protein complexes that all propagate transcription initiation (Fig. 1B). For PGC-1α, this assembly has been studied in great detail: The AD

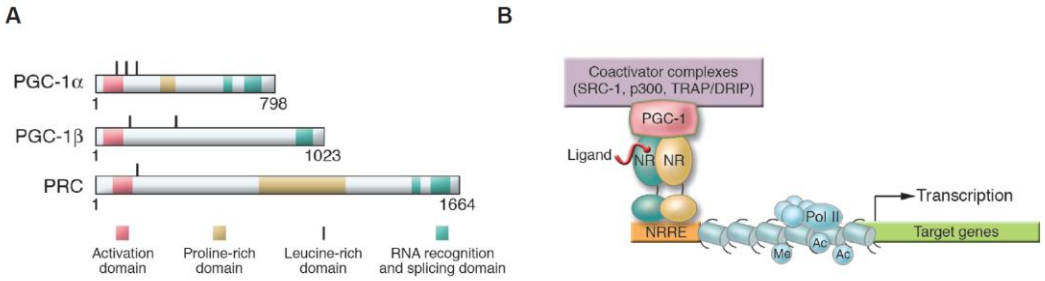


**Figure 1** ***PGC-1 structure and function*** **A.** Domain structure of PGC-1 family members. **B.** Schematic representation of transcription initiation facilitated by PGC-1**.** Adapted from (3).

interacts with the histone acetyltransferases (HATs) steroid receptor-1 (SRC-1) and cyclic AMP response element-binding protein (CREB) binding protein (CBP)/p300, which mediate decondensation of chromatin (1). BRG-1-associated factor (BAF) 60a recruits the chromatin remodelling switch/sucrose nonfermentable (SWI/SNF) complex to a central stretch within the PGC-1α protein (2), while the C-terminal region binds the thyroid receptor-associated protein (TRAP)/vitamin D

receptor-interacting protein (DRIP) or Mediator complex that directly associates with the basal transcription machinery and thus contributes to preinitiation complex formation (4).

As PGC-1 does not contain a DNA-binding domain, PGC-1-mediated gene expression depends on its affinity to certain transcription factors. First described as a coactivator of PPARγ and the thyroid hormone receptor (TR) (5), PGC-1α was subsequently show to interact with most nuclear receptors (NRs) including PPARα (6), PPARβ/δ (7), liver X receptor (LXR) (8), farnesoid X receptor (FXR) (9), vitamin D receptor (VDR) (10), constitutive androstane receptor (CAR) (11), pregnane X receptor (PXR) (12), estrogen receptor (ER) α (13), estrogen-related receptor (ERR) α and γ (14), glucocorticoid receptor (GR) (15), hepatic nuclear factor (HNF) 4α (15,16), and retinoid X receptor (RXR) (17). PGC-1β also coactivates PPARα (18), ERα (19), and ERRγ (20), while both PGC-1β and PRC are able to enhance ERRα-dependent transcription (21,22).

Binding of PGC-1 to NRs can be either constitutive or ligand-dependant, and indeed, human PGC-1α was identified in a screen for steroid-responsive genes (23). Although the three dimensional structure of none of the PGC-1 coactivators has been resolved yet, N-terminal fragments of PGC-1α were crystallized in complex with ERRα (24), ERRγ (25), PPARγ (26), and HNF4α (27) highlighting the requirement of the LXXLL motifs as crucial NR binding interfaces. These leucine-rich domains are also essential for the interaction of PGC-1β with ERα (19). Contact to a NR causes a conformational change in PGC-1α, which compacts its AD and thus favours interaction with HATs (25). Therefore, the transcription activation potential of PGC-1 depends on its association with NRs. This association can be prevented by competition for binding sites on NRs resulting in diminished target gene expression. Corepressors that were shown to act as such competitors and displace PGC-1 include small heterodimer partner (SHP) on GR (28), SHP interacting leucine zipper protein (SMILE) on ERRγ (29) and dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on X chromosome, gene 1 (DAX-1) on ERRγ and HNF4α (30,31).

PGC-1α is also able to coactivate some non-NR transcription factors like forkhead box protein (Fox) O1 (32), FoxO3a (33), nuclear respiratory factor (NRF) 1 (34), myocyte-specific enhancer factor (MEF) 2A (35), and MEF2C (36). They bind PGC-1α at domains different from NRs and the presence of leucine-rich motifs for this interaction is dispensable. NRF-1 can also be coactivated by PRC (37) while PGC-1β is an important cofactor for sterol regulatory element-binding protein (SREBP) 1a and 1c (38), and FoxA2 (39,40). Beyond the initiation of RNA polymerisation, PGC-1α is also implicated in RNA processing *via* the RNA recognition and splicing domain at the C-terminus (41).

Several isoforms of PGC-1α have been identified: The naturally occurring NT-PGC-1α arises from alternative splicing and contains amino acids 1-270 (42). Compared to the full-length form, NT-PGC-1α is more stable and localized in the cytoplasm due to continuous exported from the nucleus (43). Furthermore, alternative promoter usage produces 2 isoforms termed PGC-1α-b and PGC-1α-c (PGC-1α-a denotes the full-length form) that play a role in rodent and human skeletal muscle (44,45).

A liver-specific isoform (L-PGC-1α) that lacks amino acids 1-127 is only present in humans (46). Analogous to PGC-1α, transcription of PGC-1β may also start from two different promoters giving rise to the PGC-1β-1 and PGC-1β-2 isoforms, while alternative splicing of exon 11 generates PGC-1β-a and PGC-1β-b respectively (47). No isoforms of PRC are known so far.

### 1.1.2 Expression and function of PGC-1 coactivators

PGC-1α orthologues are present in all vertebrates species, with 95% sequence homology between mouse and human, and a high degree of conservation in the AD and NR interaction domain due to their importance in mediating PGC-1 action (23,48). In *Drosophila*, the PGC-1 homologue Spargel is essential in mitochondrial genes expression and *spargel* mutants display respiration defects (49). This fundamental role of PGC-1 in cellular respiration is conserved across species, redundant between all 3 family members and important in all tissues that express PGC-1. Therefore, the coordination of oxidative metabolism is the central property of this coactivator family. PGC-1α and PGC-1β are consequently enriched in tissues with high oxidative capacity like skeletal muscle, heart, brown adipose tissue (BAT), kidney, and brain (5,18) whereas PRC is expressed ubiquitously (37).

Mitochondria are the main site of oxidative energy production and comprise 2 membrane layers, the inner and the outer mitochondrial membrane. The inner membrane is folded into cristae enlarging its surface, and contains proteins which engage in oxidative phosphorylation to supply the cell with ATP (50). The inner space or mitochondrial matrix harbours enzymes of β oxidation, pyruvate decarboxylation and the tricarboxylic acid (TCA) cycle as well as mitochondrial DNA. To produce ATP from nutrients, on the one hand, lipids are activated in the cytosol and shuttled into mitochondria as acylcanitines. Mitochondrial β oxidation then generates acetyl-CoA which is fed into the TCA cycle (50). On the other hand, carbohydrates are converted into pyruvate by glycolysis in the cytosol. Upon import into mitochondria, pyruvate is transformed into acetyl-CoA by pyruvate decarboxylation. Using acetyl-CoA as substrate, the TCA cycle through a series of reactions then provides NADH which acts as electron donor in the electron transport chain (ETC). The electrons from NADH are transferred between different donor and acceptor proteins (complex I – IV) to finally reduce oxygen to water (50). During this electron passage energy is liberated and stored as an electrochemical proton gradient across the inner mitochondrial membrane. Dissipation of this gradient finally drives ATP production by ATP synthase (50).

Increasing the number of mitochondria to meet higher energy demands is a complex process that requires a high degree of coordination as it involves transcription of nuclear genes, import of the resulting proteins into mitochondria, transcription of mitochondrial DNA and its replication. PGC-1 integrates these steps by coactivating the nuclear transcription factors NRF-1 and ERRα on promoters

of nuclear encoded mitochondrial genes (Fig. 2) (21,22,34,37,51). These include cytochrome c oxidase subunit 4 (COX IV) which forms a part of the ETC, β-ATP synthase, as well as the transcription factor A, mitochondrial (Tfam). Tfam governs transcription and replication of mitochondrial DNA yielding mitochondrial encoded parts of the ETC and the genetic material required for mitochondrial biogenesis. In addition to coactivation, PGC-1α also induces the expression of NRF-1, NRF-2 and ERRα (Fig. 2) (34,52). NRF-2 and ERRα further amplify their own as well as each others expression thus constituting a powerful positive regulatory mechanism for oxidative phosphorylation (53). Yet, mice with a global deletion of either PGC-1α (54,55) or PGC-1β (56,57) are viable, fertile and do not show overtly deranged mitochondria. Their ability to cope with different stress conditions (for example exposure to cold) is however grossly compromised. This indicates that PGC-1 coactivators are not required for mitochondrial function in the basal state, but if the cell needs to meet higher energy demands, PGC-1 coactivators are crucial to coordinate the program of mitochondrial biogenesis.

The coupling of electron transport and production of ATP is not complete and protons may leak over the inner mitochondrial membrane thereby generating heat. Ectopic expression of PGC-1α and PGC-1β increases mitochondrial proteins and respiration to a similar degree, but proton leak in cells expressing PGC-1α is higher (58). Thus PGC-1α and PGC-1β have shared but also distinct properties.

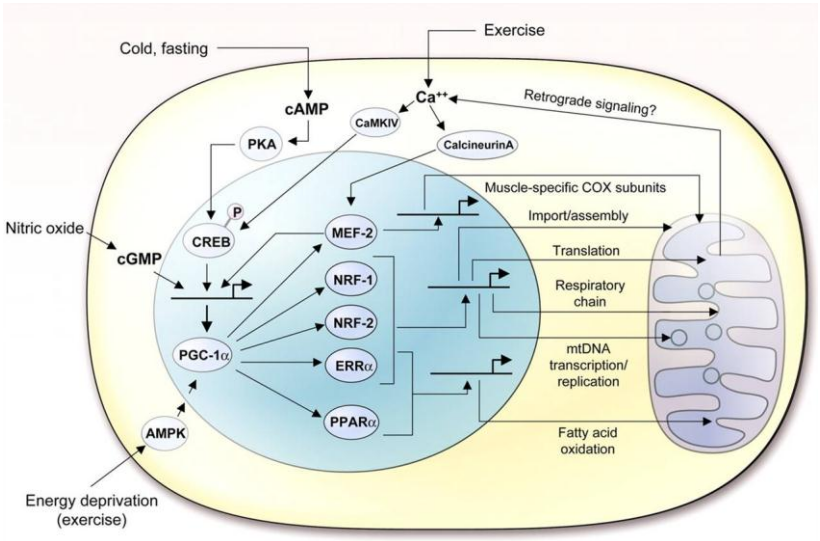


**Figure 2** ***Schematic representation of PGC-1α´s role in coordinating oxidative metabolism.*** From (59).

Reactive oxygen species (ROS) are free radicals that arise as byproducts from the ETC when oxygen is prematurely reduced. As highly reactive molecules, they have the capacity to damage components of the cell and therefore, ROS levels need to be tightly controlled. An increase in mitochondrial biogenesis is inevitably accompanied by an increase in ROS production. To alleviate

this oxidative stress, PGC-1α and PGC-1β enhances the expression of ROS detoxifying enzymes like glutathione peroxidise 1 (GPx-1) and superoxide dismutase [Mn], mitochondrial (Sod2). Along these lines, mice carrying a PGC-1α deletion are more susceptible to neurodegeneration caused by oxidative stressors while PGC-1α protects neurons from ROS-induced cell death (60). One of the transcription factors that partners with PGC-1α to coordinate ROS detoxification is FoxO3a. While both are activated by high ROS levels, FoxO3a also induces PGC-1α transcription. This signal amplification with positive feedback loop finally ensures non-toxic ROS levels in the cell (33).

Beyond oxidative phosphorylation itself, PGC-1 coactivators also augment substrate availability. Both, PGC-1α and PGC-1β coactivate ERRα and PPARα, master regulators of mitochondrial fatty acid oxidation, in a constitutive and a ligand-dependant manner, respectively (6,61,62). The induction of key enzymes in β oxidation like medium-chain specific acyl-CoA dehydrogenase (MCAD) and carnitine O-palmitoyltransferase (CPT) 1 allows the cell to rely mainly on fatty acids as energy substrate (Fig. 2). This notion is extended as PGC-1α was shown to also induce peroxisome biogenesis, an organelle that participates in the catabolism of very long and branched chain fatty acids (63). Accordingly, PGC-1 coactivators co-ordinately heighten a whole program of oxidative metabolic pathways turning them into chief mediators of cellular energy metabolism.

Besides their role in respiration, PGC-1 coactivators also execute an array of tissue-specific functions. In liver, PGC-1α is markedly upregulated upon fasting downstream of glucocorticoids and the glucagon/cAMP/CREB axis (64) regulating several important starvation pathways. These include gluconeogenesis and ketogenesis (16), the former being induced by PGC-1α-mediated coactivation of HNF4α and FoxO1, which leads to the expression of major gluconeogenic enzymes like phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) (15,32). Under the control of the same transcription factors, selenoprotein P (SeP) expression in liver is also governed by PGC-1α illustrating its important role in selenium homeostasis (65). PGC-1α has been further implicated in hepatic heme and bile acid biosynthesis through the induction of 5-aminolevulinate synthase (ALAS-1) and cholesterol 7-α-hydroxylase (CYP7A1) respectively (66,67). Finally, regulation of homocysteine homeostasis has been assigned to PGC-1α (68). By contrast, PGC-1β is not able to induce gluconeogenesis due to lack of binding to HNF4α and FoxO1 (62). Yet, PGC-1β plays an important role in hepatic lipid metabolism by its ability to stimulate lipogenic gene expression through coactivation of SREBP-1a and SREBP-1c (38). In concert with FoxA2, PGC-1β also promotes mitochondrial β oxidation and triglyceride/VLDL secretion into plasma thus reducing accumulation of fat in the liver (40). Interestingly, SREBP-1 may also interfere with the HNF4α/PGC-1α interaction and as a result suppress gluconeogenesis (69). Therefore, PGC-1α and PGC-1β have distinct functions in liver but the pathways they regulate intersect at different levels and mutually influence each other.

In the heart, PGC-1α induction coincides with perinatal mitochondrial biogenesis which causes a switch in fuel usage from glucose to fatty acids and occurs after fasting when ATP is needed. Hearts from mice with PGC-1α deletion fail to augment cardiac output in response to chemical or electrical stimulation while cardiac stress and aging lead to heart failure (70,71). Comparably, lack of PGC-1β accelerates heart failure (72), but in both cases the phenotype only manifests under stress conditions. By contrast, animals with double deficiency die shortly after birth with small hearts that are defective in maturation keeping a fetal gene expression pattern (73). This illustrates that both coactivators can partly compensate for each other in the heart. Notably, cardiac PGC-1α transgenic animals lose the sarcomer structure in the heart and develop dilated cardiomyopathy (74). The cardioprotective function of PGC-1α is therefore confined to a certain therapeutic window.

Originally, PGC-1α was discovered as a thermogenic regulator in BAT induced upon exposure to cold (5). The main function of BAT is the production of heat by uncoupled respiration while white adipose tissue (WAT) stores energy in the form of lipid droplets. The relevance of both PGC-1α and PGC-1β in BAT was confirmed *in vitro* and *in vivo* with double deletion models that exhibited severe defects in mitochondrial function and density (73,75). Moreover, PGC-1α and PGC-1β are crucial in BAT differentiation because they coactivate PR domain zinc finger protein (PRDM) 16 on promoters of brown adipocyte lineage genes whereas C-terminal binding protein (CtBP) prevents recruitment of PGC-1α/β on promoters of white adipocyte lineage genes (76). Hence, suppression of PGC-1α e.g. by twist-1 or retinoblastoma proteins favours a white over a brown fat phenotype (77,78). On the other hand, induction of PGC-1α in WAT e.g. by fibroblast growth factor (FGF) 21 leads to "browning" characterized by the expression of genes like UCP-1 that are able to dissipate energy (79,80). In light of metabolic diseases that arise from obesity, this property of PGC-1 coactivators is of prime interest. A recent mouse model with an adipose tissue-specific deletion of PGC-1α confirms this concept because the animals develop insulin resistance on a high fat diet due to high circulating lipid levels (81).

In pancreas, both PGC-1α and PGC-1β are invovlved in glucose-stimulated insulin secretion from β cells; the precise role of both coactivators is however controversial as suppression and induction of insulin release have been reported (39,82,83).

Additive and independently, PGC-1α and PGC-1β control mitocondrial density in neurons and, as mentioned above, PGC-1α acts neuroprotective under conditions of oxidative stress through its ability to detoxify ROS (60,84). Its beneficial effect was further shown in the context of amyotrophic lateral sclerosis (ALS) (85) whereas suppressed levels of PGC-1α have been reported in different neurodegenerative disorders like Huntington´s disease (86) and Alzheimer´s diesase (87). Consistently, global and brain-specific deficiency of PGC-1α causes striatal degenerative lesions that dysregulate systemic energy balance (54,88). Therefore, PGC-1 is not only implicated in peripheral but also in central regulation of energy metabolism.

Finally, PGC-1α has been associated with retinal susceptibility to light damage (89) and chondrogenesis (90) while osteoclast activation is coordinated by PGC-1β (91). A non-redundant function in proliferative growth has been assigned to PRC (92). The role of PGC-1 coactivators in skeletal muscle will be discussed in more detail below (see 1.1.4).

### 1.1.3 Modulation of PGC-1 activity

PGC-1 coactivators translate various environmental stimuli into adaptive, transcriptional programs in different tissues. These external cues therefore induce PGC-1 expression through several molecular cascades. The signalling network involved has been best studied for PGC-1α and will be described in the following. CREB is a potent inducer of PGC-1α expression in different tissues (64) and binds to cAMP response elements (CRE) in the promoter region after being phosphorylated. Protein kinase A (PKA) can mediate this phosphorylation e.g. upon cold exposure and subsequent β3-adrenergic signalling in BAT or after glucagon stimulation in liver (93). In skeletal muscle, calcium/calmodulin-dependent protein kinase type (CaMK) IV is able to phosphorylate CREB and augment PGC-1α transcription. CaMK IV activation occurs after exercise-mediated calcium release. Free, cytosolic calcium also induces calcineurin A (CnA) which enhances MEF2C and MEF2D binding to MEF2 sites in the PGC-1α promoter. As PGC-1α coactivates MEF2 on these sites, a positive regulatory loop controls PGC-1α expression (94). Negative regulation occurs via the recruitment of histone deacetylase (HDAC) 5 to MEF2 (95). Another important kinase in PGC-1α transcription is p38/MAPK (96). Activated in response to exercise or PKA signalling, p38/MAPK targets MEF2 and activating transcription factor (ATF) 2, which both bind to their cognate response elements in the PGC-1α promoter and enhance transcription (97). As sensor of low energy levels in the cell, AMP-activated protein kinase (AMPK) is also active upon muscle contraction and promotes PGC-1α expression (98). An autoregulatory loop as described above for MEF2 also exists for PPARs: PPARγ activation induces PGC-1α which coactivates PPARγ on PGC-1α´s own promoter in adipocytes (99). In skeletal muscle, PPARβ/δ is both a target of PGC-1α and coactivated by it on the PGC-1α promoter (99). Finally, PPARα governs the determination of brown adipocytes by inducing PRDM16 which in turn coactivates PPARα on the PGC-1α promoter (100). Positive feed-forward mechanisms are thus common in the induction of PGC-1α offering the possibility of signal amplification and fine-tuned expression.

Repression of the PGC-1α gene has also been described: Activation of protein kinase B (PKB)/Akt upon insulin stimulation leads to phosphorylation of FoxO1 resulting in dissociation from insulin response elements (IREs) in the PGC-1α promoter and hence diminished expression (101).

Furthermore, corepressor recruitment to the promoter suppresses PGC-1α transcription. Examples are SHP binding to ERRγ decreasing energy production in BAT (102) and nuclear receptor corepressor (NcoR) 1/HDAC3 docking to Rev-erbα to block heme biosynthesis (103). A more general mechanism of repression is hypermethylation of non-CpG nucleotides in the PGC-1α promoter which is caused by DNA methyltransferase (DNMT) 3B and reduces mitochondrial content (104).

Besides changes in PGC-1 expression levels, the regulation of protein stability is of fundamental importance. Different post-translational modifications (PTMs) of PGC-1α and PGC-1β that influence coactivator potential have been reported. These include phosphorylation, acetylation, methylation, GlcNAcylation, sumoylation and ubiquitylation. Phosphorylation can occur at several serine and threonine residues either enhancing or repressing PGC-1α activity (Fig. 3). Being a short-lived and aggregation-prone protein (105), PGC-1α is stabilized by p38/MAPK phosphorylation (Fig. 3) (106) and the interaction with the repressor p160 myb binding protein (p160 mbp) decreased resulting in transcriptional activation (107). As p38/MAPK also targets PGC-1α expression, this kinase acts in a dual mode of action with a fast (direct phosphorylation) and a sustained (induction of

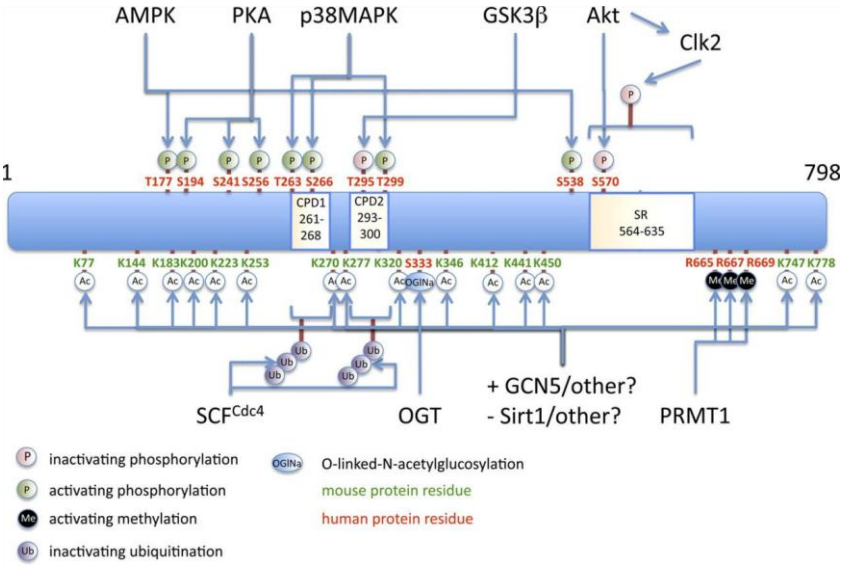


**Figure 3** ***Post-translational modifications of PGC-1α.*** From (110).

expression) effect. Likewise, AMPK phosphorylates PGC-1α (Fig. 3) while at the same time spurring its expression (98). For NT-PGC-1α, a similar mechanism was found: PKA-mediated phosphorylation (Fig. 3) diminishes nuclear export resulting in enhanced nuclear availability and stronger coactivation potential (43). Simultaneously, PKA activates CREB. This double regulation also exists reciprocally for PGC-1α deactivation. While Akt causes FoxO1 release from the PGC-1α promoter, it also phosphorylates PGC-1α directly (Fig. 3) to inhibit its recruitment to transcription factors (108). Another negative phoshphorylation is mediated by glycogen synthase kinase (GSK) 3β (Fig 3) (109).

A second PTM that impacts on PGC-1α activity is acetylation. The acetyltransferase general control of amino acid synthesis (GCN) 5 acetylates PGC-1α at multiple lysine residues (Fig. 3) rendering it inactive due to relocalisation to nuclear foci (111). Acetylation by GCN5 was also shown to repress PGC-1β activity (112). Opposed to GCN5, NAD-dependent protein deacetylase sirtuin (Sirt) 1 removes acetyl residues from PGC-1α (Fig. 3) resulting in higher transcription activation potential (113,114). As Sirt1 is an energy sensor, this is an important mechanism to secure cellular energy supply. Moreover, Sirt1 acts in concert with AMPK to control the energy status of the cell by co-ordinately activating PGC-1α (115).

Polyubiquitylation targets proteins for proteasomal degradation which determines their stability within the cell. Skp1/Cullin/F-box cell division control 4 ($SCF^{Cdc4}$) was shown to be a PGC-1α E3 ubiquitin ligase (Fig. 3) and their interaction requires prior negative phosphorylation e.g. by GSK-3β (116). Ubiquitylation then leads to proteasomal degradation of PGC-1α in the nucleus thus abrogating its transcription activation capacity.

Another negative PTM is SUMOylation at lysine 183 albeit without changing subcellular localisation or stability of PGC-1α (117). Presumably, SUMOylation enables a stable interaction with receptor-interacting protein (RIP) 140, a known repressor of PGC-1α activity (118).

Finally, PGC-1α is activated by O-linked beta-N-acetylglucosylation (Fig. 3) (119) and arginine methylation by protein arginine methylatransferase (PRMT) 1 (Fig. 3) (120). The many possible PTMs and various combinations thereof show that a complex network converges on PGC-1α to tightly control its activity.

### 1.1.4 PGC-1 coactivators in skeletal muscle physiology

Skeletal muscle is built up of several muscle bundles called fasciculi that are surrounded by connective tissue (epimysium) and attached to the bone by tendons (Fig. 4A). Each bundle comprises parallel running myofibers. These myofibers are postmitotic, syncytial cells that are formed by the fusion of mononucleated myoblasts (muscle stem cells / satellite cells) which line muscle fibers and retain the potential to regenerate injured muscle cells (121). Each myofiber contains numerous myofibrils (Fig. 4A). Myofibrils are linear sequences of sarcomers, the fundamental contractile unit of skeletal muscle. The appearance of these sarcomer repeats as distinct brighter and darker bands under the microscope gives skeletal muscle its striated pattern (121). Sarcomers are composed of the interdigitating myofilaments myosin and actin with actin being anchored in the Z disk (Fig. 4A). For contraction, these two filaments slide along each other thus shortening the muscle (Fig. 4B). Contraction is initiated after motor neuron excitation which results in an action potential and finally release of acetylcholine into the synaptic cleft at the neuromusulcar junction. This depolarizes the

muscle membrane (sarcolemma) at the motor end plate and causes a muscle action potential that propagates along the fiber (121). The sarcolemma has deep invaginations (transverse tubules) which lie in close proximity to the intracellular sarcoplasmic reticulum (Fig. 4A). Upon depolarization, voltage-gated dihydropyridine receptors in the sarcolemma of transverse tubules directly couple to and activate ryanodine receptors in the sarcoplasmic reticulum, which consequently release calcium into the cytoplasm. Free calcium then enables the sarcomer to contract (Fig. 4B). This occurs through binding of calcium to troponin thus changing the conformation of the troponin-tropomyosin complex that winds around actin and prevents myosin binding in the basal state (Fig. 4B 1). Upon calcium binding, the active sites on actin are exposed and the myosin heads form cross bridges (Fig. 4B 2) which subsequently move, sliding the filaments against one another (Fig. 4B 3). The tight link between electrical stimulation and muscle contraction is called excitation-contraction coupling. The interaction of myosin and actin is released by binding of ATP to the myosin head enabling another cycle of contraction (Fig. 4B 4). Contraction is terminated when calcium is pumped back into the sarcoplasmic reticulum (Fig. 4B 5) (121).

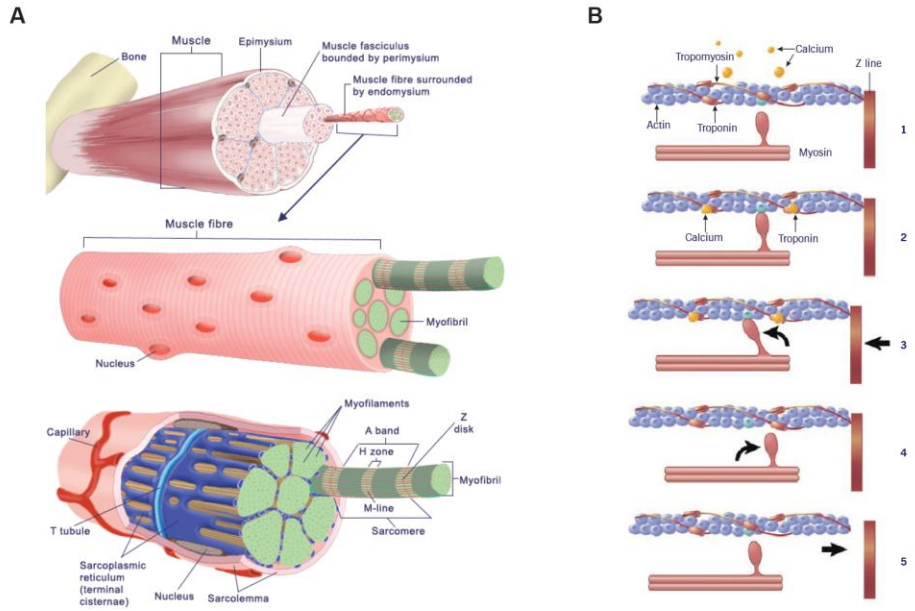


**Figure 4** ***Skeletal muscle structure and function***. **A.** Organization of skeletal muscle in fasciculi (bundles), myofibers, myofibrils and sarcomers. Adapted from (122). **B.** Stages of muscle contraction according to the sliding filament theory. From (123).

Skeletal muscle is a plastic organ and myofibers can be distinguished by the type of myosin they express. Type I or slow fibers appear red, are highly resistant to fatigue but produce relatively low maximal force while type II fibers appear white, fatigue rapidly but produce high maximal force (121). The latter can be further subdivided into type IIa (moderately fast), and type IIx (fast) fibers in humans, while rodents also posses type IIb (very fast) fibers. Corresponding to the different properties,

type I and IIa fibers rely on oxidative metabolism as energy source and exhibit high mitochondrial density while type IIx and IIb fibers mainly depend on glycolcytic metabolism (121).

PGC-1α is abundant in skeletal muscle and especially enriched in slow / oxidative muscle fibers that contain high numbers of mitochondria. Forced expression converts white muscles with mainly fast fibers into red muscles with properties of slow type I and IIa fibers (124). PGC-1α is thus sufficient for a slow fiber type switch. PGC-1β on the other hand was shown to drive the formation of type IIx fibers that however still posses a relatively high oxidative capacity (125). In agreement with its role in oxidative myofiber determination, PGC-1α is transiently induced in skeletal muscle after a single bout of endurance exercise and chronically elevated in endurance trained muscle both in rodents and humans (126-128). This induction is caused by pathways that are active in contracting skeletal muscle including calcium signalling, p38/MAPK and AMPK (see 1.1.3) and in large part due to an increase in the alternative isoforms PGC-1α-b and PGC-1α-c (44). Elevated PGC-1α levels in turn mediate the major adaptation that skeletal muscle undergoes in response to endurance training (Fig. 5).

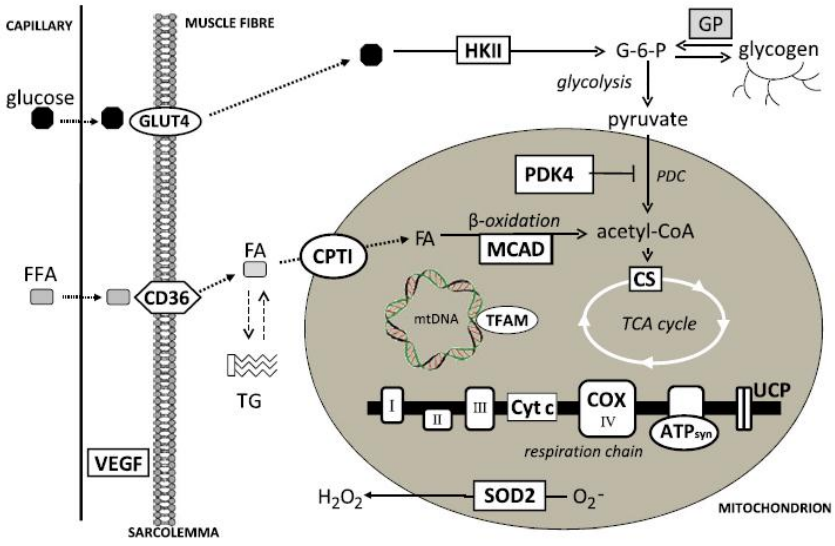


**Figure 5** ***Schematic overview of pathways and the most important proteins regulated by PGC-1α in skeletal muscle.*** From (130).

These concern substrate supply, utilization, energy generation and protection of muscle fibers from damage. More precisely, formation of a larger proportion of oxidative fibers requires mitochondrial biogenesis which is coordinated by PGC-1α through induction of Tfam and nuclear encoded subunits of the ETC (Fig. 5 and see 1.1.2). During exercise, the initial phase of mitochondrial biogenesis is mediated by an activation of PGC-1α protein followed by a second phase characterized by elevated PGC-1α gene expression (129).

To secure cellular energy supply under conditions of continuous muscle contraction, the induction of mitochondria is accompanied by an induction of enzymes involved in fatty acid uptake

and oxidation like CD36, CPT1 and MCAD (Fig. 5 and see 1.1.2) (131). In addition, expression of the glucose transporter GLUT4 is elevated by PGC-1α *in vitro* (36) and *in vivo* (131) resulting in higher glucose transport into the muscle cell (Fig. 5). At the same time, PGC-1α mediates a rise in pyruvate dehydrogenase kinase (PDK) 4 which inhibits the pyruvate dehydrogenase complex (PDC) thus shutting down glucose oxidation (Fig. 5) (132). Therefore, PGC-1α favours fatty acids as energy substrate coherent with their role in aerobic exercise. Excess glucose that cannot be diverted through glycolysis, is then stored in form of glycogen (Fig. 5) (131,133). As higher mitochondrial activity is accompanied by the production of larger amounts of ROS, which are potentially detrimental for the cell, PGC-1α also acts on ROS detoxifying enzymes like Sod2 (Fig. 5) thus protecting the cell from oxidative damage (134). Moreover, ROS were shown to be important for the induction of PGC-1α thus forming a negative feedback loop (135). Apart from metabolic fluxes within the cell, PGC-1α ensures tight linkage between muscle and nerve through regulation of genes of the neuromuscular junction and promotion of acetylcholine receptors clustering at the motor end plate (136). Furthermore, PGC-1α potently induces vascular endothelial growth facto (VEGF) (137). VEGF mediates angiogenesis thus maintaining sufficient nutrient and oxygen supply to muscles during endurance exercise (138). In conjunction, all these PGC-1α-mediated changes enable muscle cells to adapt to endurance training. Functionally, this translates into a higher endurance capacity of PGC-1α skeletal muscle-transgenic mice (MCKα) (133) whereas the skeletal muscle-specific deletion of PGC-1α (MKO) deteriorates performance (139). Inflammatory changes in that latter model will be discussed below (see 1.3).

In contrast to PGC-1α, PGC-β is not uniformly influenced by exercise. Still, when overexpressed in skeletal muscle, genes involved in oxidative phosphorylation and fatty acid oxidation are upregulated resulting structurally in higher mitochondrial density and functionally in a better endurance performance (125). Inversely and resembling the MKO model, skeletal muscle-specific PGC-1β deletion causes lower endurance capacity (140). A combined global deficiency of PGC-1α with muscle-specific gene ablation of PGC-1β aggravates this phenotype with a very severe exercise deficit, strongly reduced oxidative capacity and mitochondrial structure and function derangements. However, fiber type determination is only slightly changed towards more type I fibers (140). Like PGC-1α, PGC-1β was also shown to induce angiogenesis (141). It therefore appears that PGC-1α and PGC-1β share some properties in skeletal muscle. The role as "exercise factor" is however unique to PGC-1α and such a clear function could not be assigned to PGC-1β so far.

Apart from the regulation of endurance adaptations, PGC-1α was also shown to be a target of the myogenic regulatory MyoD in terminal muscle differentiation (142). Furthermore, PGC-1α has an impact on the circadian rhythm by stimulating clock genes thus integrating the cell-autonomous clock and energy metabolism (143). Interestingly, MyoD itself is a target of circadian transcription activators and their disruption perturbs muscle function as well as PGC-1α and PGC-1β expression (144).

Further studies are needed to investigate the mutual dependencies of PGC-1 coactivators, MyoD and clock genes to define their contribution to skeletal muscle physiology.

### 1.1.5 PGC-1 coactivators in skeletal muscle pathology

Skeletal muscle plasticity does not only comprise the ability to adapt to different kinds of training (strength versus endurance) with respective enrichment of type II or type I fibers, but also the capacity to match different amounts of use. While endurance training induces PGC-1α, muscle disuse or unloading e.g. through denervation results in muscle wasting (atrophy) with markedly decreased PGC-1α and PGC-1β levels (145,146). Concomitantly, the ubiquitin ligases atrogin-1 and muscle-specific RING finger protein (MuRF) 1 are induced mediating higher protein degradation rates that are characteristic for the atrophic state. Overexpression of PGC-1α and PGC-1β is able to suppress both atrogin-1 and MuRF-1 resulting in lower protein degradation rates and thus rescues muscle cells from atrophy *in vitro* and *in vivo* (147,148). Mechanistically, the suppression of FoxO3 transcriptional activity by both coactivators is responsible for diminished atrogen expression. Low levels of PGC-1α have further been shown in a number of muscle wasting conditions like diabetes, uremia and cancer cachexia (148) as well as after statin treatment which is used against hypercholesterolemia and induces muscle breakdown as a side effect. This statin-induced muscle damage can also be prevented by PGC-1α overexpression (149). It is therefore evident, that PGC-1α and PGC-1β are able to preserve muscle structure and function under conditions where these are usually lost. This observation extends to sarcopenia denoting progressive muscle loss with age. Old mice with skeletal muscle-specific overexpression of PGC-1α perform better in exercise tests than old wild-type mice exhibiting improved mitochondrial function and a higher degree of overall muscle integrity (134).

Besides its beneficial effect in muscle wasting, PGC-1α also conveys improved phenotypes in some inherited muscle disorders. First, Duchenne muscular dystrophy (DMD) is caused by an X-linked mutation in dystrophin which anchors the cytoskeleton in the extracellular matrix. The mutation renders the protein defective thus leading to degeneration of muscles and premature death. Skeletal muscle-specific overexpression of PGC-1α improves symptoms of sedentary as well as exercised *mdx* mice (a mouse model for DMD) with markedly decreased fiber damage (136). Second, mitochondrial myopathies stem from mutations in mitochondrial DNA generating deficits in energy metabolism. Again, skeletal muscle-specific overexpression of PGC-1α ameliorated muscle function with higher ATP levels and delayed onset of disease (150). A similar effect could be achieved by endurance exercise (151). Third, Huntington´s disease (HD) is characterized by weight loss due to loss of muscle mass early on in HD etiology. In mouse models of HD and HD patients, PGC-1α was found to be repressed in skeletal muscle. Overcoming this repression by ectopic expression of PGC-1α from an adenoviral vector improves pathology in the mouse model (152). Targeting PGC-1α and PGC-1β

pharmacologically or increasing PGC-1α by endurance training when possible thus represents a promising treatment strategy in a number of skeletal muscle disorders.

Apart from muscle disorders, metabolic diseases are linked to dysregulation of PGC-1 coactivators. Insulin resistance is a hallmark of type 2 diabetes and arises from overnutrition with constant oversupply of fatty acids that mitochondria do not accomplish to oxidize completely. Therefore, lipid metabolites like diacylglycerol accumulate within muscle cells activating serine kinases like protein kinase C (PKC) and others. These kinases phosphorylate insulin receptor substrate (Irs) 1 at inhibitory serine residues thus blocking tyrosine phosphorylation upon insulin binding to the insulin receptor. This in turn impairs phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) and downstream Akt activation which blunts GLUT4 recruitment to the membrane. Consequently, insulin-stimulated glucose uptake into the muscle is diminished contributing to hyperglycaemia in diabetes.

Obesity is a common risk factor for diabetes and both genetic and acquired obesity lead to downregulation of PGC-1α and PGC-1β in skeletal muscle of rodents, presumably due to high levels of saturated free fatty acids (FFAs) (153). Furthermore, a coordinate suppression of genes involved in oxidative phosphorylation has been observed in human diabetic patients alongside a reduction of both PGC-1α and PGC-1β levels in some, but not all studies (154-156). Along these lines, a moderate increase of PGC-1α in skeletal muscle improves insulin sensitivity by selectively augmenting fatty acid oxidation in subsarcolemmal but not intermyofibrillar mitochondria (157). The relation between PGC-1 coactivators and insulin resistance is however more complex. Mice from two independent lines with a global PGC-1α deletion are less susceptible to diet-induced obesity and stay lean due to profound hyperactivity caused by striatal lesions in the brain (54,55). To circumvent this phenotype, a model with PGC-1α deletion exclusively in skeletal muscle has been developed. Yet, these mice display increased peripheral insulin sensitivity even though they become glucose intolerant on a high fat diet because of muscle-islet crosstalk: Muscle-derived interleukin 6 (IL-6) likely impairs insulin secretion from pancreatic β cells. The lack of insulin then prevents sufficient uptake of glucose into the muscle (158). Inversely, MCKα mice also exhibit a complex phenotype as they are more prone to diet-induced insulin resistance than wild-type mice. The reason for inhibited insulin signalling presumably is an increased content of intracellular lipids which accumulate because of unbalanced lipid supply that exceeds mitochondrial fatty acid oxidation capacity (159). During aging however, this same model preserves insulin signalling and is protected from age-associated insulin resistance (134). Correspondingly, in a twin study, both PGC-1α and PGC-1β were determined to be reduced by age representing an explanation for an increased risk of diabetes at older age (160). This decline is further associated with the common Gly482Ser variant in PGC-1α. Genetic linkage studies that tried to establish a relationship between this polymorphism and diabetes however yielded inconsistent results (161,162) and no functional consequence of the serine form is detectable (163). Analogous, a variant of PGC-1β (Ala203Pro) was associated with obesity in one study (164) but protection from diabetes in another study (165). In contrast, polymorphisms in the promoter of the PGC-1α gene were shown to

contribute to diabetes in Koreans and Austrians (166,167). Concerning PGC-1β, transgenic expression protects mice from diet-induced and genetically determined obesity (21), however, no direct influence on glucose metabolism is evident as global deletion of the protein does not affect whole body glucose or insulin tolerance on high fat diet (56), nor does a hypomorphic mutation influence these parameters or muscle glucose uptake (168). Both strains do however develop hepatic insulin resistance due to accumulation of lipids in the liver. Surprisingly, the double deletion of PGC-1α and PGC-1β does not have an impact on glucose or insulin tolerance neither on chow nor on high fat diet (HFD). As the model employs the global PGC-1α deletion as background for muscle-specific PGC-1β gene ablation, the same concerns (cerebral lesions) apply and weight gain between the groups might be different possibly explaining the phenomenon (140).

On the contrary, treatment of mice with resveratrol protects them from diet-induced obesity and insulin resistance. This effect is due to higher oxidative capacity of their muscle fibers induced by Sirt1-mediated PGC-1α deacetylation and consequently higher PGC-1α activity (169,170). The insulin-sensitizing properties of thiazolidinediones are also partially ascribed to an induction of PGC-1α and the concomitant restoration of mitochondrial bioenergetics in muscle e.g. in db/db mice (171). It therefore appears that targeting PGC-1α can be beneficial to counteract metabolic diseases. The therapeutic window of PGC-1α induction is however somewhat narrow and an overkill of PGC-1α in muscle acts rather detrimental. Dysregulation of PGC-1α in other organs that point in the opposite direction e.g. excess PGC-1α in liver and pancreas in diabetes further complicate treatment strategies aimed at PGC-1α (172).

## 1.2. Inflammation

### 1.2.1 Molecular mechanisms of inflammation: the NF-κB pathway

Inflammation has evolved to protect the organism from harmful insults by initiating an immune response that drives defence and repair mechanisms of the body, and is characterized by redness (rubor), swelling (tumor), heat (calor), and pain (dolor) due to higher blood flow to the injured site. During the course of an inflammation, a complex interplay between cells of the injured tissue and cells of the innate and adaptive immune system orchestrates clearance of pathogens and wound healing. Innumerous signals participate in this process depending on the site and degree of injury, the pathogen(s) involved, the infection history of the individual etc. In this context, cytokines play an important role, as they mediate cell-cell communication and are able to elicit, potentiate or dampen an inflammatory reaction thus exerting major influence on the progression of the inflammatory reaction.

At the molecular level, the NF-κB pathway is central to inflammatory processes. Induced by various external stimuli and therefore downstream of different cell surface receptors, signals converge

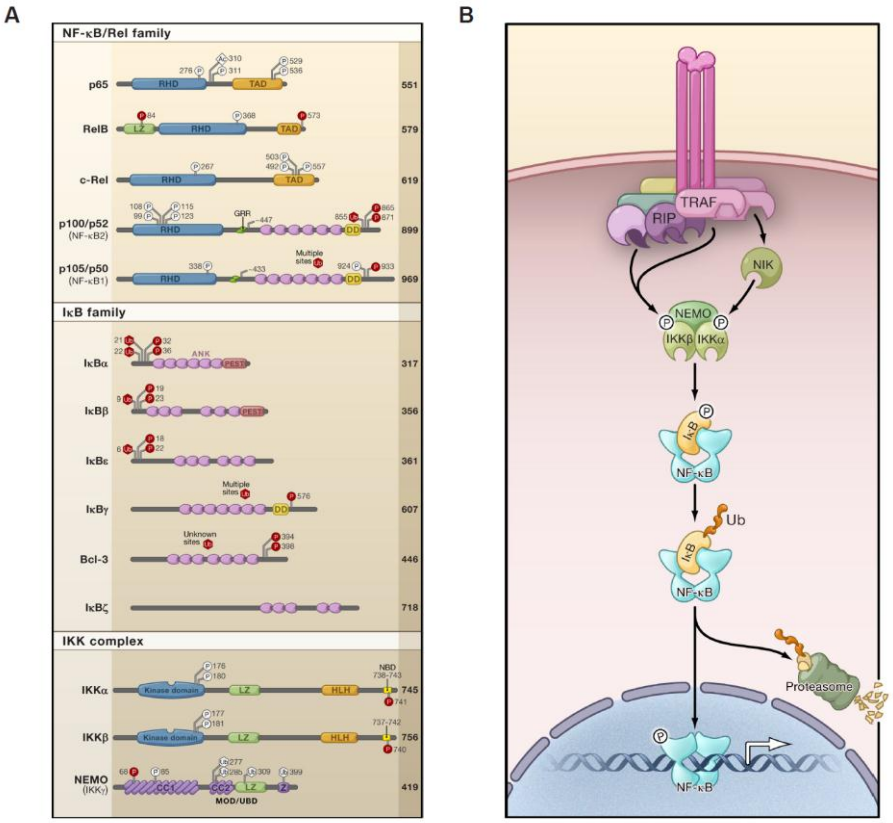


**Figure 6** ***The NF-kB pathway.*** **A.** Members of the NF-κB, IκB and IKK family, their domains and selected PTMs. **B.** Signalling cascade of the NF-κB pathway. Adapted from (173).

at inhibitor of κB (IκB) kinase (IKK) phosphorylation (Fig. 6A and B). Activated IKK subsequently phosphorylates IκB, which forms a cytosolic complex with an NF-κB dimer. Phosphorylation-induced polyubiquitylation targets IκB for proteasomal degradation consequently releasing NF-κB and allowing for nuclear translocation (173). In the nucleus, NF-κB then binds to κB sites and initiates transcription of pro-inflammatory cytokines (Fig. 6B). As IKK, IκB and NF-κB all constitute protein families with several members, a multitude of different signalling possibilities abounds. The NF-κB family comprises 5 members, namely RelA/p65, RelB, c-Rel, p100/p52 and p105/p50 that all share an N-terminal Rel homology domain (RHD) (Fig. 6A). These subunits form homo-and heterodimers that bind to DNA at the κB consensus site 5´-GGGRNWYYCC-3´ (where N is any base, R is purine, W is adenine or thymine, and Y is pyrimidine). Only p65, RelB and c-Rel additionally contain a C-terminal transactivation domain (TAD) which enables them to initiate transcription once bound to DNA, whereas p50 and p52 as homodimers act repressive as they lack this TAD (173). The most common and best studied dimer is p65/p50.

The IκB family comprises the typical members IκBα, IκBβ and IκBε and the atypical members IκBγ, B-cell lymphoma (Bcl) 3, and IκBζ which all possess several ankyrin repeat domains (Fig. 6A). The typical members sequester NF-κB dimers in the cytosol thus preventing transcription from κb site in unstimulated cells (174,175). Upon stimulation, the typical IκBs are degraded at different rates, whereby IκBα has the fastest kinetics (176,177). This leads to exposure of the nuclear localization sequence on NF-κB dimers and their entry into the nucleus where they drive transcription (Fig. 6B). As the IκBs are also NF-κB target genes, their resynthesis terminates the signal representing a negative feed-back loop (178). Again, IκBα, IκBβ and IκBε display different resynthesis kinetics (179).

The atypical IκBs all act in the nucleus by binding to NF-κB dimers and thus have quite different functions from typical IκBs. They confer either inhibitory or activating properties to the dimers they bind to, causing potentiation or repression of transcription. Bcl-3 contains a TAD (in contrast to other IκBs) and interacts with p50 or p52 homodimers in the nucleus. This enables transcription driven by otherwise repressive NF-κB dimers (180). However, Bcl-3 might also displace p50 and p52 homodimers from DNA thus allowing active NF-κB forms to bind to these sites or stabilize the repressive forms on DNA with the opposite effect (181). These contradictory functions of Bcl-3 depend on the cellular context and are likely regulated by PTMs. Outside of the NF-κB pathway, Bcl-3 also acts as cofactor for other transcription factors. IκBζ is induced by some, but not all stimuli of the NF-κB pathway and associates mainly with p50 homodimers. Although IκBζ does not contain a TAD, it transactivates p50 homodimers while inhibiting p65-containing NF-κB complexes (182). The precursor proteins p100 and p105 can also be classified as IκBs as they contain ankyrin repeats domains and serve the purpose of preventing NF-κB dimers from entering the nucleus (183). While constitutive processing of p100 and p105 yields the NF-κB subunits p52 and p50,

respectively (184,185), unprocessed p100 associates with p65/p50 dimers acting as classical IκB, which is entirely degraded upon stimulation (186). Both, p100 and p105 also form dimers with various NF-κB subunits and retain them in the cytoplasm: RelB selectively binds to p100, and induced processing to p52 generates the RelB/p52 dimer that has an important function in the alternative pathway (see below). Likewise, p105 associates with cytosolic p65 or c-Rel and prevents nuclear entry. Stimulus-dependent processing of p105 then yields the p65/p50 or c-Rel/p50 dimers that translocate to the nucleus and activate transcription (187). Finally, an alternative transcript of the p105 gene gives rise to IκBγ whose role is not yet defined (188).

The IKK family consist of the three members IKKα, IKKβ and IKKγ/NF-κB essential modulator (NEMO) (Fig. 6B). IKKα (189) and IKKβ (190) are closely related and both contain a kinase domain rendering them catalytically active while NEMO is structurally unrelated and acts as regulatory subunit (191). Two major pathways have been described in NF-κB signalling: the canonical and the noncanonical or alternative pathway. Canonical signalling leads to activation of the heterotrimeric IKK complex consisting of all 3 IKK subunits. IKKβ-mediated phosphorylation of IκBα subsequently frees p65/p50 that further acts as transcription factor in the nucleus (192). While IKKα is dispensable for canonical signalling, it is required for noncanonical NF-κB activation (193). Thereby and independent of NEMO, IKKα dimers phosphorylate p100 which complexes RelB. Processing to p52 then results in formation of the RelB/p52 dimer that translocates to the nucleus and induces a subset of NF-κB target genes (194). The multitude of possible interaction within the NF-κB pathway illustrates the complexity of inflammatory signalling but at the same time allows a specific and appropriate reaction to different kinds of injury and infection.

Upstream of IKK, various receptors transmit signals that lead to NF-κB activation: Cytokines produced either by cells of the immune system or by cells from an affected tissue signal *via* cytokine receptors. Tumor necrosis factor (TNF) α (195) is one such cytokine that has a well-established role in inducing inflammatory reactions and binds to TNF receptor (TNFR) 1 and TNFR2 (196). Upon stimulation TNFR1 oligomerizes and recruits TNFR-associates death domain protein (TRADD) (197) *via* death domain (DD) interaction. TRADD acts as a platform to assemble the NF-κB-activating complex. Therefore, TNFR-associated factor (TRAF) 2 (197), the kinase receptor interacting protein (RIP) 1 (198) and the ubiquitin ligases inhibitor of apoptosis (cIAP1 and cIAP2) (199) sequentially bind to TRADD. Lysine 63-linked (200) ubiquitylation of RIP1 then facilitates recruitment of transforming growth factor (TGF) β activating kinase (TAK) 1 and IKK resulting in IKK activation by TAK-1-dependent and transautophosphorylation (Fig. 6B) (201). In the absence of cIAP 1/2, TNFR1 may trigger apoptosis via recruitment of Fas–associated DD (FADD) and caspase-8 (197). TNFR2 ligation also causes oligomarization but the receptor does not contain a DD (202). Therefore, direct association with TRAF2 results in the recruitment of the components of the IKK activating complex (RIP1, cIAP, TAK1). TNFR1 and TNFR2 are both also able to induce activator protein (AP) 1 signalling via c-Jun N-terminal kinase (JNK) (203).

Receptors that recognize conserved bacterial or viral structures, so-called pathogen-associated molecular patterns (PAMPs) belong to the toll-like receptor (TLR) family. So far, 12 different mammalian TLRs have been identified with the following specificities: TLR2 forms heterodimers with TLR1 or TLR6 recognizing bacterial lipoproteins. TLR4 in conjunction with CD14 reacts to lipopolysaccharide (LPS), TLR5 to flagellin and TLR9 to unmethylated CpG all characteristic of bacterial pathogens. As these are mostly outside of the cell, the corresponsing TLRs are located on the cell surface (except for TLR9). TLR3, TLR7 and TLR8 recognize viral RNA (TLR3: double stranded, TLR7 and TLR8: single stranded) and are therefore located within the cell (204). Upon ligation, TLRs recruit adaptor proteins *via* Toll IL-1R (TIR) domains. For most TLRs, these adaptors are myeloid differentiation primary response gene (Myd) 88 and Myd88 adaptor-like protein (Mal) (205,206), which in turn associate with IL-1R-asscoiated kinase (IRAK) 4 that further binds IRAK1 (206). This complex is responsible for phosphorylation of TRAF6 (207), which finally leads to the TAK1 and IKK recruitment and subsequent activation (208). Downstream of TLR3 and TLR4, TIR domain-containing adaptor molecule (TRIF) and TRIF-related adaptor molecule (TRAM) act as adaptor proteins to induce TRAF3-dependent interferon expression *via* interferon regulatory factor (IRF) 3 (209-211). TRIF may also activate RIP1 and thus induce the NF-κB pathway (209). Free fatty acids share structural similarity with bacteria-derived ligands and therefore activate TLR2 and TLR4 in the absence of pathogens (212,213). As FFAs are elevated in plasma of obese individuals, this aberrant activation represents one link between metabolic dysregulation and inflammatory signalling.

In lymphocytes, the alternative NF-κB pathway has been characterized downstream of CD40 (214), lymphotoxin β receptor (LTβR) (215), B-cell activating factor receptor (BAFFR) (216) and receptor activator of NF-κB (RANK) (217). These signals lead to activation of NF-κB inducing kinase (NIK), which in turn phosphorylates IKKα thus initiating p100 processing and noncanonical signalling (Fig. 6B).

Another layer of complexity is added to NF-κB signalling by PTMs. Due to its particular importance, p65 has been studied extensively in this regard and inducible phosphorylation as well as acetylation (218), ubiquitylation (219), prolyl isomerisation (219), poly(ADP-ribosyl)ation (220), monomethylation (221) and S-nitrosylation (222) were reported. These PTMs affect subcellular localization, the stability of DNA-bound NF-κB dimers or their ability to interact with coactivators and corepressors thus fine-tuning inflammatory responses. Phosphorylation of p65 at serine 276 within the RHD is crucial for the recruitment of CBP/p300 and the ensuing transcriptional activation (223). PKA (223) and mitogen- and stress-activated protein kinase (MSK) 1 (224) can phosphorylate this residue upon stimulation whereas a mutation to alanine abrogates the inducible expression of a subset of target genes (225). In turn, this phosphorylation is a prerequisite for acetylation of lysine 310, which also facilitates transcription by recruiting HATs and displacing HDACs from NF-κB (226). IKKα (227), IKKβ (228,229), IKKε (230), TRAF family member-associated NF-κB activator (TANK) binding kinase (TBK) 1 (230) and ribosomal S6 kinase (RSK) 1 (231) phosphorylate p65 at serine 536

within the TAD. Analogous to serine 276, serine 536 phosphorylation propagates CBP/p300 association resulting in augmented transcription while mutation to alanine abolishes this effect and reduces p65 acetylation at lysine 310 (232). In this state, corepressors like NCoR1 or silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) preferentially bind to p65 and inhibit transcription. Importantly, changes in transcriptional activation through shifted coactivator/corepressor balance can occur without alterations in DNA binding of NF-κB. Transcriptional activity is further modulated by inducible phosphorylation of serine 529 within the TAD by casein kinase (CK) II (233) and serine 311 within the RHD by PKCζ (234). Several other serine and threonine residues within p65 can be phosphorylated, the functional consequences of these PTMs however remain poorly defined to date.

### 1.2.2 Inhibition of inflammation: interference with the NF-κB pathway

Inflammatory reactions are transient responses that protect against injury and infection but if rendered chronic, they in fact become detrimental to the organism. Therefore, tight control mechanisms govern inflammation and may inhibit the process at several levels. Concerning the NF-κB pathway, interference with the signalling cascade can occur during virtually all steps. Some important negative regulators of NF-κB will be discussed in the following section grouped by their mode of action.

Enrichment of typical IκB proteins (see 1.2.1) fosters the retention of NF-κB dimers in the cytosol thus blocking nuclear entry and binding to DNA which blunts pro-inflammatory gene expression. The same applies to p100 and p105 elevation as they bind cytosolic NF-κB subunits; however, the effect depends vitally on the specificity of this binding because an elevation of RelB/p52 for example displaces p65-containing complexes from κB sites (235). As the different dimers do not have the same transcriptional activation capacity on a promoter, this might decrease target gene expression but can also sustain the response as RelB/p52 is resistant to IκB resynthesis (235). Induction of atypical IκBs can also inhibit pro-inflammatory gene expression by stabilizing repressive p50 or p52 dimers on DNA thereby blocking access of activating dimers to κB sites (181,182,236). These effects are target gene-specific and therefore always affect only a subset of NF-κB inducible genes.

Ubiquitylation has a dual function in NF-κB signalling: Lysine 48-linked polyubiquitylation targets proteins for proteasomal degradation. This might initiate target gene expression as in case of IκBs or terminate the signal e.g. through degradation of p65 (237). Ubiquitylation of p65 is mediated by suppressor of cytokine signalling (SOCS) 1 whose overexpression blocks IL-1β-dependent NF-κB activation (219). In contrast, regulatory lysine 63-linked ubiquitylation activates distinct steps in the

signalling cascade and therefore regulates target gene expression positively. A20 is an ubiquitin editing enzyme and A20-deficient mice develop severe inflammation (238). That is due to the ability of A20 to remove regulatory lysine 63-linked ubiquitin modifications from RIP1 and instead attach lysine 48-linked degradative ubiquitin chains which terminates TNFR signalling (239). A similar mechanism has been reported downstream of TLRs where A20 removes regulatory ubiquitylation from TRAF6 again shutting down the signal (240).

Multiple phosphorylation events are part of NF-κB activation and therefore, kinase dysregulation may impede signalling. By blocking access of the IKK complex, β-arrestin inhibits phosphorylation of IκBα and subsequently its degradation thus diminishing NF-κB activation (241). Further downstream, kinases that mediate p65 phosphorylation (see 1.2.1) can be modulated. Among these, MSK1 is regulated by p38. MSK kinase (MK) 2 and its substrate heat shock protein (Hsp) 27 sequester p38 in the cytosol thus preventing p65 hyperphosphorylation (242). Moreover, Akt signalling has been implicated in inflammatory gene expression downstream of TNFR (243). The effect of Akt on NF-κB activation is dependent on both IKKα and IKKβ, and on serine 536 within the TAD of p65 (244,245). As canonical signalling relies on IKKβ, IKKβ-deficient MEFs can neither phosphorylate IκBα nor p65 while IKKα-deficient MEFs normally degrade IκBα but are still unable to phosphorylate p65 which blunts transcription from NF-κB-inducible promoters (245). Although phosphorylation of p65 appears critical in NF-κB activation and several kinases that target p65 have been identified, knowledge about phosphatases that remove this modification is scarce. Inhibition of protein phosphatase (PP) 1 and PP2A by calyculin A (246) or okadaic acid (OA) (247) leads to an accumulation of serine 536-phosphorylated p65 in the basal state while inducible serine 536 phosphorylation cannot be released under these conditions. Therefore, PP1 and PP2A target p65 but their substrate specificity is rather broad. The only p65-specific serine phosphatase discovered so far is Wip1. Wip1 dephosphorylates p65 at serine 536 and mice lacking this phosphatase display increased NF-κB target gene expression alongside higher p65 phosphorylation levels (248).

The importance of p65 phosphorylation derives from the ability of the phosphoprotein to recruit transcription coactivators. The exchange of corepressors for coactivators depends on transducin β-like protein 1X (TBL1)/TBL-1-related protein (TBLR) 1 which mediate binding of the ubiquitin-proteasome complex to corepressor followed by their degradation (249). Sustained binding of corepressor like SMRT to p65 conversely inhibits transcription of pro-inflammatory factors (250) as SMRT recruits HDAC3. This interaction is disrupted if IKKα phosphorylates SMRT (232). Furthermore, competition for coactivators has been proposed as model to limit transcriptional activation. For example, p53 also employs CBP/p300 from a limited pool decreasing the chances of p65 to interact with CBP (251). This transcriptional crosstalk is further refined by IKKα (252) and GSK3β (253).

Lastly, transrepression through NRs is a potent mechanism to dampen inflammatory gene expression. Activation of PPARα was accordingly shown to reduce expression of IL-6,

cyclooxygenase (COX) 2, vascular cell adhesion molecule (VCAM) 1 and other inflammatory factors in different cell types (254,255) while mice with a PPARα deletion react hypersensitive to LPS (256). Similarly, PPARγ ligands strongly inhibit expression of pro-inflammatory mediators (257,258) and glucocorticoids that signal *via* GR exert well-established anti-inflammatory effects (259,260). In contrast to NR transcriptional activation, transrepression does not require DNA binding, and the pathways targeted (NF-κB and AP-1) are not interrupted upstream but modulation commonly occurs in the nucleus at the level of DNA-bound transcription factor.

Several molecular mechanisms of transrepression were established so far, some of which resemble inhibitory processes also employed by other factors that are described above. First, NRs may induce IκBα (Fig. 7a) in a DNA-binding dependent or –independent manner as shown for PPARα thus terminating NF-κB dependent gene expression (261-264). Second, competition for a limiting pool

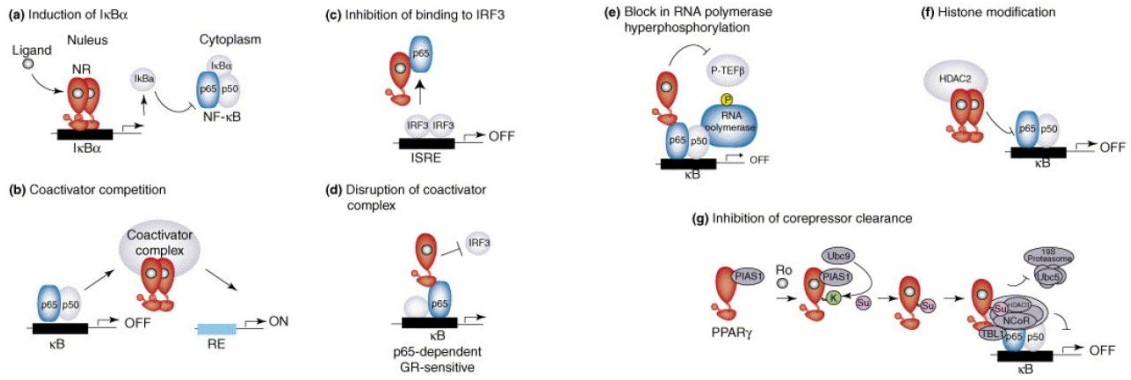


**Figure 7** ***Molecular mechanisms of transrepression***. Adapted from (265).

of coactivators like for example CBP (Fig. 7b) can entice these coactivators away from NF-κB and result in mutual antagonism between inflammatory and NR pathways (266,267). Coactivators are also able to directly recruit NRs to NF-κB resulting in transrepression as reported for GR and nuclear TR-interacting protein (nTrip) 6 (268). Third, direct interaction between NRs and inflammatory transcription factors can interfere with activation of pro-inflammatory promoters hindering DNA-binding of NF-κB or AP-1 (269). In particular, downstream of TLR4, IRF3 requires p65 as transcription cofactor that can be tethered by GR (Fig. 7c) (270,271). IRF3 might conversely act as a cofactor for p65 and this interaction is also sensitive to GR blockade as direct binding of GR to p65 precludes IRF3 recruitment (Fig. 7d) (271). GR transrepression can be potentiated by secondary recruitment of cofactors like glutamate receptor-interacting protein (GRIP) 1 (272); binding of NUR-related factor (Nurr) 1 to p65 and further recruitment of CoREST constitutes a similar mechanism limiting inflammatory gene expression in microglia and astrocytes (273). Competition between GR and the transcription elongation factor P-TEFb explains the repressive effect of GR on the IL-8 promoter. Although RNA polymerase II is recruited, GR prevents phosphorylation of the C-terminal

domain of RNA polymerase II which is a prerequisite for elongation (Fig. 7e) (274,275). Forth, chromatin remodelling plays a role in transrepression as reported for the IL-1β promoter. GR recruits HDAC2 to NF-κB in this promoter fostering corepressor complex stabilization and transcriptional repression (Fig 7f) (276,277). Finally, inhibition of corepressor clearance from DNA-bound NF-κB constitutes a complex mechanism of transrepression: In the basal state, NF-κB is occupied by a corepressor complex that undergoes proteasomal degradation upon stimulation and further replacement by coactivaotrs. Proteasomal degradation of this corepressor complex can be inhibited by PPARγ and LXRs. This requires ligand-dependent SUMOylation which enhances the affinity of PPARγ or LXRs for NcoR/HDAC resulting in decreased inflammatory gene expression (Fig. 7g) (278,279). LXRs are also implicated in transrepression of signal transducer and activator of transcription (STAT) 1 downstream of interferon signalling (280). All the abovementioned mechanisms are examples of transrepression. A unified model that would explain under which conditions different NRs act transrepressive, how different stimuli (like TNFα or TLR ligation) contribute and how target genes-specificity is achieved still needs to be developed.

### 1.2.3 Macrophage polarization

Macrophages are cells of hematopoietic origin that serve vital functions in tissue homeostasis and immune defence. Their precursors develop in the bone marrow from hematopoietic stem cells through multipotent progenitor stages committing to the myeloid lineage. Under the influence of macrophage colony-stimulating factor (MCSF), these progenitors differentiate into monoblast, and further into pro-monocytes and monocytes that leave the bone marrow to become circulating peripheral blood mononuclear cells (PBMCs) (281). Under homeostatic conditions, PBMCs invade different organs and terminally differentiate thus replenishing the pool of tissue-resident macrophages (282). These may be activated in response to different signals like injury or infection. During an inflammation, more PBMCs are attracted to the inflamed site by chemokines and differentiate into inflammatory macrophages that support clearance of pathogens and tissue debris by phagocytosis (281,282). They further contribute to the inflammatory process by secreting specific cytokines. There is evidence that at least two subsets of circulating monocytes exist which differ in their expression of surface markers like lymphocyte antigen (Ly) 6C (282). How these monocyte subsets give rise to different macrophage population is however not fully understood yet.

Macrophage diversity is evident from their appearance in different tissues, e.g. Kuppfer cells in liver, alveolar macrophages in the lungs and microglia in the nervous system. Further plasticity abounds in immune activation with two types of macrophages generally distinguishable from one another: classically and alternatively activated cells, also referred to as M1 and M2 macrophages. This

classification follows the Th1/Th2 paradigm established in T cell biology reflecting a highly inflammatory, cytotoxic or a immunomodulatory, humoral immune response, respectively (283). M1 macrophages are therefore primed by interferon (IFN) γ (284) and further activated by cytokines like TNFα or pathogenic signals like LPS that signal *via* TLRs (285). This classical activation augments phagocytic activity leading to engulfment of pathogens and necrotic cells. Concomitantly, production of toxic intermediates like nitric oxide (NO) and ROS is increased to effectively destroy pathogens within phagolysosomes (286). High antigen presentation capacity complements the ability of these M1 cells to sustain an adaptive type 1 immune reaction. This is further fostered by the cytokine profile they start to express: High levels of IL-12 contribute to Th1 polarization while neutrophils, natural killer cells, and activated T cells are attracted by chemokines like macrophage inflammatory protein (MIP) 1α/CCL3, CXCL10 or IL-8 (285). The pro-inflammatory environment created at the site of infection is preserved by secretion of TNFα, IL-6 and IL-1β while the release of proteolytic enzymes facilitates degradation of extracellular matrix components (281,285). Therefore, permanent M1 macrophages activation may lead to profound tissue damage.

Conversely, M2 macrophages are activated in a Th2 cytokine environment. Originally characterized in response to treatment with IL-4 as macrophages that are less inflammatory than those activated by IFNγ (287), alternative activation became an umbrella term for several forms of non-M1 cells. They all share low IL-12 and medium to high IL-10 expression but other than that have quite divergent traits. Several criteria to subdivide M2 macrophages have been proposed either according to function (host defence, wound healing and immune regulation) (281) or according to stimulus (282,285). First, macrophages that are activated in response to IL-4 or IL-13 are termed M2a. They secrete low levels of pro-inflammatory cytokines like TNFα, IL-6 or MIP-1α but high levels of anti-inflammatory IL-10, IL-1 receptor antagonist (IL-1Ra) and decoy IL-1RII. Moreover, release of the chemokines CCL17, CCL18, CCL22 and CCL24 attracts cells involved in type 2 immune responses like Th2 cells, regulatory T cell, basophils and eosinophils (288-290). Intracellularly, arginase is induced which metabolizes arginine to ornithin (a precursor of polyamines) and inhibits NO production thus ensuring efficient wound healing and helminthic parasite clearance (286). Second, macrophages that are activated in response to immune complexes and TLR or IL-1R ligation are termed M2b (291). These cells retain higher levels of pro-inflammatory cytokines (TNFα, IL-6, IL-1, but not IL-12) alongside a potent IL-10 and a selective CCL1 release (292). CCL1 acts on Th2 and regulatory T cells as well as eosinophils that all express CCR8. M2b macrophages therefore play a predominantly immunoregulatory role (293). Third, macrophages activated by IL-10 are termed M2c. They share low pro-inflammatory cytokine expression and arginase induction with M2a cells and further propagate the IL-10 signal. IL-10 has an important role in uncoupling chemokine receptors which renders M2c macrophages unresponsive to chemokines and converts them into a mode of deactivation (294). Their capacity to act on B cells is caused by expression of CXCL13 thereby

favouring humoral immunity, while release of TGFβ and matrix components like versican and pentaxin-related protein PTX3 promotes tissue remodelling and repair (295,296).

The dichotomy of M1 and M2 is a useful model but does not entirely reflect the *in vivo* situation. M1 and M2 are probably extremes of a continuous spectrum of possible activation states and therefore, intermediate forms exist. Plasticity may even be retained in a way that macrophages reconvert their phenotype depending on the cytokine environment although these phenomena are poorly understood so far (297). It is known however that M2 cytokines (IL-4, IL-10, IL-13) suppress M1 activation by interfering with NF-κB and STAT1 signalling (298) e.g. through upregulating inhibitory p50 dimers (299) and STAT3/STAT6. SOCS proteins are critically involved in this cross-talk with SOCS3 polarizing towards M1 and SOCS1 towards M2 macrophages (300,301). Yet another determinant of macrophage polarization is Krüppel-like factor (KLF) 4 which cooperates with STAT6 to induce M2 and inhibit M1 genes consequently being merely present in M1 cells (302). Analogous, KLF2 impairs NF-κB activation in macrophages (303). Myeloid deletion studies revealed that both PPARγ (304) and PPARβ/δ (305,306) are important M2 drivers and recently, c-myc was shown to induce PPARγ, STAT6 and some M2 genes making it an important mediator of alternative activation (307). On the cell surface, M1 and M2 cells preferentially express different receptors that can be exploited experimentally to discriminate between the two activation types including Fc-RI, II and III (CD16, CD32, CD64), C68, CD80 and CD86 for M1 and scavenger receptors like Msr-1 and CD163, mannose receptor (MRC1/CD206), and CD14 for M2 macrophages (285).

Excessive M1 and M2 activation have both been linked to different pathologies: Tumour tolerance (308), allergy and asthma (309) are related to M2 activation whereas unresolved M1 activation leads to chronic inflammation and tissue damage as observed in autoimmune disease like rheumatoid arthritis (310). The detrimental effect in obesity will be discussed in the next chapter. During skeletal muscle repair and regeneration, macrophages also play an important role with an early M1 and a delayed M2 wave (311). The $CD68^+/CD163^-$ M1 cells readily remove cellular debris by phagocytosis directly after injury and activate satellite cells while $CD68^-/CD163^+$ M2 macrophages invade the tissue later (311). They promote differentiation of satellite cells and muscle growth to restore muscle function (312). The M1/M2 switch is presumably orchestrated by muscle-derived IL-4 (313). In dystrophic muscle, M1 activation and concomitant M2 repression e.g. by IFNγ exacerbates muscle wasting in DMD (314). Conversely, IL-10-mediated M2 activation and M1 repression alleviates symptoms (315).

### 1.2.4 Inflammation, obesity and insulin resistance

Obesity as defined by a body mass index (BMI) >30 has become a global epidemic with 500 million affected and numbers still on the rise according to the World Health Organization. The principle reason for this development is an increasingly sedentary lifestyle with a caloric intake that exceeds energy expenditure by far. Evolutionary, the human body was optimized to survive times of famine and therefore, efficient energy storage was vital which nowadays causes severe public health problems. Obesity and its sequelae like insulin resistance, type 2 diabetes, fatty liver disease, atherosclerosis, hypertension and stroke, commonly subsumed as the metabolic syndrome, are the leading cause of mortality in developed countries. Insulin resistance is thereby especially important as insulin coordinates glucose and fatty acid uptake into skeletal muscle and adipose tissue, respectively, with lipolysis in adipose tissue and gluconeogenesis in liver thus determining circulating concentrations of glucose and fatty acids. Apart from a mere dysregulation of metabolism, an immune component to overweight and obesity has been appreciated for decades as patients possess higher levels of circulating pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12, and TNFα), chemokines (monocyte chemoattractant protein (MCP) 1/CCL2, RANTES/CCL5, MIP-1), acute phase proteins (C-reactive protein (CRP), serum amyloid A, ferritin), adipokines associated with insulin resistance (retinol binding protein 4 and resistin), and procoagulative and hypertensive factors (plasminogen activator inhibitor 1 and angiotensinogen) constituting a chronic, low-grade inflammation. Reciprocally, negative acute phase proteins (transcortin and transferrin) and adipokines associated with insulin sensitivity (adiponectin, visfatin, omentin, and vaspin) are lowered in plasma of obese subjects (316).

As expansion of adipose depots is the prime effect of obesity, an inflammatory reaction in WAT is the major source of elevated circulating cytokines and the driver of insulin resistance (317). Mechanistically, inflammation impinges on metabolism at the level of Irs1, the downstream effector of the insulin receptor which is tyrosin-phosphorylated in response to insulin stimulation (Fig. 8A and see 1.1.5). This activation can be prevented by inhibitory serine phosphorylation through kinases involved in inflammatory signalling like IKK and JNK (Fig. 8A). Therefore, JNK deletion ameliorates insulin resistance and improves insulin receptor signalling in obese animals (318). A multitude of stimuli feeds into the NF-κB and AP-1 pathway, several of which are aberrantly induced in WAT of obese subjects thereby evoking IKK/JNK activation. First, cytokines may promote these pathways and indeed, treatment of adipocytes with TNFα directly leads to inhibitory Irs1 phosphorylation (Fig. 8A) (319), while animals with disrupted TNFα signalling are protected from obesity-induced insulin resistance (320). Second, high levels of saturated FFAs bind TLR4 due to structural similarity with bacterial ligands inducing IKK and JNK (Fig. 8A and see 1.2.1). Consequently, TLR4 deletion protects from obesity-induced insulin-resistance (212). Third, ER stress is a result of overnutrition, as the capacity of adipocytes to store fatty acids in form of triglycerides is exceeded and FFAs leak into

the cytoplasm in turn activating JNK (Fig. 8A) (321). Forth, the expansion of adipocytes to store increasing amounts of fat outbalances angiogenesis leading to tissue hypoxia and hypoxia-inducible factor (HIF) 1α expression (322). HIF-1α itself induces pro-inflammatory cytokine expression and thus IKK/JNK activation as do, fifth, increased levels of ROS that derive from hyperactive NADPH

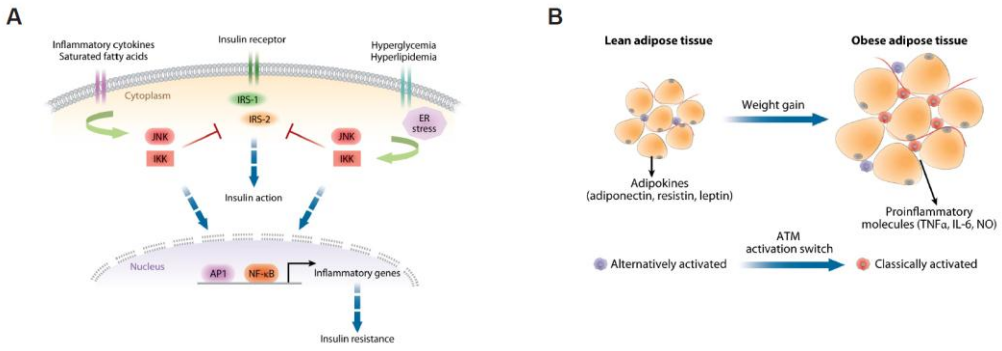


**Figure 8** ***Inflammation and insulin resistance in obesity*** **A.** Inhibition of Irs signalling by inflammatory kinases that are activated in adipocytes by different signals in obesity **B.** Schematic representation of the ATM switch from M2 in lean to M1 macrophages in obese adipose tissue. Adapted from (316).

oxidase (323). In conjunction, endoplasmic reticulum (ER) stress and hypoxia bring about adipocyte necrosis. The dying cells then nucleate the formation of crown-like structures, i.e. areas of necrotic cells surrounded by macrophages which are absent in lean animals (324).

In lean animals, adipose tissue macrophages (ATMs) are polarized towards an M2 phenotype (Fig. 8B and see 1.2.3) with anti-inflammatory properties that preclude M1 activation. It is not completely clear how this alternative activation is driven, but several nonexclusive possibilities have been studied: Adipocytes from lean mice release IL-13 and to a lesser extent IL-4, which locally cause M2 activation (see 1.2.3) without overspill into the circulation (306). Adiponectin from the same source has also been shown to polarize macrophages towards an M2 phenotype (325) and unsaturated FFAs prevail in lean subjects both systemically as well as in adipose tissue (326). All those cues lead directly or via STAT6 induction to activation of PPARs which promote an oxidative metabolic program in macrophages that corresponds to M2 activation (306). Accordingly, myeloid ablation of PPARγ or PPARβ/δ impairs M2 skewing and fosters an inflamed and insulin resistant environment with M1 activated macrophages (304,306). PGC-1β is further essential in driving oxidative metabolism characteristic of M2 activation as RNAi-mediated downregulation of PGC-1β attenuates the anti-inflammatory phenotype (327). Mechanistically, PGC-1β coactivates STAT6 and PPARs to initiate M2 gene expression. Therefore, in lean animals M2 macrophages are the prevalent ATMs in adipose tissue that contribute to sustaining an insulin sensitive milieu.

In contrast, highly inflammatory M1 macrophages accumulate in WAT of obese animals (Fig. 8B) (328,329). An altered adipokine expression profile, high levels of saturated FFA and the engulfment of necrotic adipocytes switch ATMs from the anti-inflammatory M2 towards the M1 phenotype (Fig. 8B) (330). These M1 cells secret large amounts of pro-inflammatory cytokines like

TNFα, IL-1β, and IL-6 thus constituting a feed-forward loop that amplifies and sustains adipose tissue inflammation, which aggravates insulin resistance and attracts more monocytes into WAT (Fig. 8B). MCP-1 plays an important role in monocyte recruitment and adipose tissue-specific overexpressing of MCP-1 renders animals insulin resistant (331,332) phenocopying the M1 accumulation during obesity. Correspondingly, deletion of the MCP-1 receptor CCR2 prevents this accumulation and rescues insulin sensitivity if animals are fed a high fat diet (333). Interfering with M1 activation by myeloid deletion of TLR4 (334), JNK (335) or IKKβ (336), also preserves insulin sensitivity under conditions of high fat feeding, importantly without differences in weight gain. This observation underlines the detrimental effect of M1 ATMs that couple nutrient excess to insulin resistance reinforcing inflammation as central mechanism in metabolic disorders.

Recent studies revealed that other cells of the immune system also contribute to both an anti-inflammatory M2 environment in lean animals and to obesity-induced inflammation. Eosinophils, and $CD4^+$ regulatory T and Th2 cells are all present in WAT of lean animals sustaining M2 macrophage polarization and insulin sensitivity while T cell balance in obsess animals tips towards Th1 cells (337-339). By virtue of IFNγ production, Th1 cells skew ATMs towards an M1 phenotype (340). Strikingly, in the course of weight gain, these Th1 cells exhibit a T cell receptor bias arguing for an antigen-specific expansion which would assume an autoantigen from WAT (338). Preceding macrophage accumulation, neutrophils (341) and $CD8^+$ effector T cells (342) can be detected in WAT. They contribute to M1 polarization while B cells that are also recruited to WAT early in obesity activate T cells, which in turn potentiate M1 polarization leading to insulin resistance (343). Hence, depletion of immune cells types associated with M1 polarization or induction of M2-sustaining immune cells ameliorates insulin resistance (337,338,343,344).

In liver, M1 activation of Kuppfer cells contributes to inflammation, hepatic insulin resistance and steatosis similar to the situation in WAT, while in skeletal muscle these processes have received less attention. Intramuscular fat depots that form in obese animals attract inflammatory macrophages similar to adipose tissue but to a much smaller extend (328). The detrimental effect of these cells on muscle insulin sensitivity was proven in obese animal models with myeloid deletion of either JNK (335) or IKKβ (336) where blockade of M1 macrophage activation preserves muscle insulin sensitivity. Depletion of $Cd11c^+$ cells that represent the main M1 macrophage subset has the same effect (345) while even lean mice devoid of myeloid PPARγ and thus impaired in M2 activation develop muscle insulin resistance which deteriorates under high fat feeding conditions (346). However, myeloid deletion of PPARβ/δ (306) or TLR4 (334) does not entail any effect on muscle insulin sensitivity implying a mechanism different from WAT. Details of this mechanism, e.g. how the M1/M2 switch in muscle is regulated and which signals from muscle cells sustain an M2 phenotype or induce M1 activation respectively are not understood yet and warrant further investigation.

## 1.3 PGC-1 coactivators and inflammation in skeletal muscle

Although substantial amounts of different NF-κB subunits are detectable in adult skeletal muscle, for a long time, NF-κB signalling was mainly associated with different muscle diseases that are accompanied by an inflammatory reaction like cachexia, muscular dystrophy, atrophy, and inflammatory myopathies (347). In all of these disorders, inflammation contributes to deterioration of muscle function and progression of the disease, also exemplified by skeletal muscle-specific overexpression of IKKβ which causes severe muscle wasting (348). Accordingly, a muscle-specific deletion of IKKβ improves muscle strength and counteracts denervation-induced loss of muscle mass while preserving fiber types (349). This model, as well as a model with p65 deletion, exhibits faster regeneration after injury (350) underlining the detrimental effect of canonical NF-κB signalling in unsound muscle.

In healthy muscle, NF-κB has been shown to influence myogenesis, reports are however conflicting as to whether NF-κB is a positive (351-353) or a negative (354-356) regulator of muscle differentiation. Suppression of myogenesis by canonical NF-κB signalling is mediated by induction and stabilization of cyclin D (357,358), inhibition of MyoD and myogenin expression (355), and Yin and yang (YY) 1-mediated silencing of myofibrillar genes (350). Conversely, an unexpected role for noncanonical NF-κB signalling was recently described *in vivo* (359): IKKα and RelB induce oxidative muscle fibrers and stimulate mitochondrial biogenesis by targeting PGC-1β but without impact on myogenesis. The physiological meaning of this mechanism still needs to be determined.

Exercise is also implicated in NF-κB activation in skeletal muscle (360,361), but the function thereof is unclear. ROS defence, post-exercise regeneration of damaged fibers or adaptations in fuel metabolism have been suggested as putative and non-exclusive NF-κB actions (362). Myokines like IL-8, IL-15 and particularly IL-6 are released from contracting skeletal muscle (363) rendering muscle an endocrine organ. The induction of IL-6 in muscle under these conditions however does not seem to be under the control of NF-κB but was speculated to depend on nuclear factor of activated T cells (NFAT) and/or p38/MAPK (364). Although IL-6 elevation is associated with chronic inflammation, transient exercise-derived IL-6 mediates some of the beneficial effects of physical activity. Therefore, IL-6 infusion increases insulin-stimulated glucose disposal in humans (365) and IL-6 deletion in mice results in mature-onset diabetes (366). As IL-6 receptor expression also increases after exercise, the beneficial versus detrimental effect of that cytokine might also be explained by “IL-6 resistance” (analogous to insulin resistance) under conditions of low-grade inflammation (367). IL-6 is further required for hypertrophic muscle growth through stimulation of satellite cell proliferation (368) as is IL-4 by promoting myoblast fusion (313). Satellite cells also attract and interact with monocytes/macrophages to foster muscle growth (369) illustrating yet another example of muscle and immune cell crosstalk.

Lack of exercises, i.e. a sedentary lifestyle, is a major risk factor for many chronic diseases including the metabolic syndrome, cardiovascular diseases, musculoskeletal and neurological disorders and even some cancers (370) while exercise capacity is a strong predictor of all-cause mortality (371). Many of these chronic diseases are correlated with a systemic, sterile, sub-acute inflammation as described for obesity and insulin resistance above (see 1.2.4). In contrast, physical activity may prevent the development and antagonize the progression of some of these diseases e.g. diabetes (372), sarcopenia (373), and neurodegeneration (374), and counteract systemic inflammation (375). Muscle-derived IL-6 thereby increases the anti-inflammatory cytokines IL-1Ra and IL-10 in blood (376) while inhibiting TNFα production (377) thus skewing immune function towards a type 2 response. Contraction also increases plasma levels of hormones like adrenalin, cortisol, growth hormone and prolactin that have an immunomodulatory function (375). On monocytes, exercise reduces TLR1, TLR2, and TLR4 expression and function, alongside a decrease in MHC II and costimulatory molecules (378). Potentiation of these effects leads to a temporary immunodepression after very strenuous training (375).

PGC-1α is a powerful mediator of training adaptation as explained under 1.1.4, but no direct action on myokine expression or immunomodulation after exercise has been reported. Inversely, a downregulation of PGC-1α and PGC-1β is evident in skeletal muscle of diabetic patients (155) that exhibit both higher levels of IL-6 and TNFα in skeletal muscle and a higher circulating IL-6 concentration. More precisely, expression levels of PGC-1α inversely correlated with IL-6 and TNFα expression independent of BMI (158) suggesting a causal relationship between PGC-1α and expression of pro-inflammatory cytokines. This is further underscored by the MKO and SKM-Het models (see 1.1.4) with complete deletion or heterozygous expression of PGC-1α in skeletal muscle respectively. Both display elevated IL-6 expression in muscle and higher IL-6 concentrations in plasma (158) reminiscent of the situation in diabetic patients. MKO muscles are further characterized by expression of a panel of pro-inflammatory markers like TNFα and CD68 (158), worsening after exercise, when expression of TNFα in muscle and as well as plasma levels are exorbitantly high (139). These observations imply that local and/or systemic cytokine levels may be (at least partially) under the control of skeletal muscle PGC-1α, but neither MCKα nor MCKβ mice have been investigated in this regard, and putative anti-inflammatory properties of PGC-1 coactivators remain elusive.

## 1.4 Hypothesis

The project presented here was designed to explore the role of PGC-1α and PGC-1β in inflammatory responses in skeletal muscle cells *in vitro* and *in vivo*. Deletion studies in rodent skeletal muscle hint at an anti-inflammatory role of PGC-1α while data from diabetic patients with chronic, low-grade inflammation denote a reduction of both coactivators in skeletal muscle pointing in the same direction. Moreover, exercises induce PGC-1α and counteract inflammation. If these effects are causal and PGC-1 coactivators possess anti-inflammatory properties is however not clear to date. To settle this question, we overexpressed PGC-1α and PGC-1β in C2C12 cells (manuscript 1) or in skeletal muscle of mice (manuscript 2) and studied the effect of different inflammatory stimuli on cytokine expression. We further investigated the mechanistic basis of anti-inflammatory PGC-1 action with special focus on the NF-κB pathway. Finally, we describe an unexpected cross-talk between myocytes and macrophages that results in M2 macrophage skewing representing a new kind of anti-inflammatory PGC-1 impact.

# 2 THE PGC-1 COACTIVATORS REPRESS TRANSCRIPTIONAL ACTIVITY OF NK-κB IN SKELETAL MUSCLE CELLS (MANUSCRIPT 1)

Petra S. Eisele[1,2], Silvia Salatino[1], Jens Sobek[3], Michael O. Hottiger[2,4], Christoph Handschin[1,2]

[1]Biozentrum, Division of Pharmacology/Neurobiology, University of Basel, Basel, Switzerland, [2]Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland,[3]Functional Genomics Center Zurich, ETH Zurich/University of Zurich, Zurich, Switzerland, [4]Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Zurich, Switzerland

**Running title:** *PGC-1s inhibit NF-κB activity in muscle*

**Background:** PGC-1α and PGC-1β are metabolic coactivators that are dysregulated in muscle in many chronic diseases.

**Results:** PGC-1α and PGC-1β differentially suppress expression of pro-inflammatory cytokines induced by various stimuli.

**Conclusion:** In muscle cells, PGC-1α and PGC-1β modulate the NF-κB pathway thus profoundly affecting inflammatory processes.

**Significance:** Targeting PGC-1α and PGC-1β in chronic diseases might reduce inflammation and thereby reverse disease progression.

**Keywords:** PGC-1; skeletal muscle; inflammation; NF-κB

**Accession Numbers** Microarray data have been deposited in the ArrayExpress database under accession number A-MEXP-1502 (array design) and E-MEXP-3676 (experimental data).

**Author contributions**: P.S.E. designed and performed all cell culture and biochemical experiments (cytokine expression and release, DNA binding and reporter gene assays, protein and phospho-protein detection, cofactor and nuclear receptor expression, inhibitor studies), prepared RNA for the microarray, analyzed the data and wrote the manuscript, S.S. did bioinformatic analysis on micorarray data, J.S. performed hybridizations and scanning of the microarray, M.O.H. supervised the design of the microarray and critically reviewed the manuscript, C.H. supervised the study and wrote the manuscript.

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## 2.1 Abstract

A persistent, low-grade inflammation accompanies many chronic diseases that are promoted by physical inactivity and improved by exercise. The beneficial effects of exercise are mediated in large part by PGC-1α while its loss correlates with propagation of local and systemic inflammatory markers. We examined the influence of PGC-1α and the related PGC-1β on inflammatory cytokines upon stimulation of muscle cells with TNFα, toll-like receptor agonists and free fatty acids. PGC-1s differentially repressed expression of pro-inflammatory cytokines by targeting NF-κB signaling. Interestingly, PGC-1α and PGC-1β both reduced phoshorylation of the NF-κB family member p65 and thereby its transcriptional activation potential. Taken together, the data presented here show that the PGC-1 coactivators are able to constrain inflammatory events in muscle cells and provide a molecular link between metabolic and immune pathways. The PGC-1s therefore represent attractive targets to not only improve metabolic health in diseases like type 2 diabetes but also to limit the detrimental, low-grade inflammation in these patients.

## 2.2 Introduction

A sedentary lifestyle is a strong and independent risk factor for a large number of chronic diseases including musculoskeletal, metabolic, cardiovascular and neurological disorders. These diseases have been linked to a sterile, persistent, low-grade inflammation with elevated levels of circulating cytokines like interleukin 6 (IL-6), tumor necrosis factor α (TNFα) and IL-1β that often worsen disease progression (1-3). The nuclear factor κB (NF-κB) pathway is a central regulator of inflammatory processes: NF-κB activation has accordingly been associated with obesity and insulin resistance in different organs (4-6). A wide array of signals including cytokines like TNFα and toll-like receptor (TLR) agonists of pathogenic or dietary origin (e.g. excess free fatty acids as in obesity (7)) is able to boost NF-κB activity upon cell surface receptor binding. These ligand-receptor interactions trigger the recruitment of adaptor proteins and receptor-proximal kinases ultimately culminating in the activation of the inhibitor of NF-κB (IκB) kinase (IKK) complex. IKK subsequently phosphorylates IκB which is then degraded by the proteasome. Decreased levels of IκB free NF-κB, thereby enabling cytosolic-nuclear translocation and ultimately transcriptional induction of a large amount of genes involved in immune function (8). The NF-κB family comprises the 5 members RelA/p65, RelB, c-Rel, p100/p52 and p105/p50, of which the heterodimer p65/p50 is the most common form and the target of so-called “classical” NF-κB activation (8). The transcriptional activity of p65 is further modulated by post-translational modification i.e. inducible phosphorylation events that affect the binding affinity to coactivators and corepressors without altering the recruitment to DNA response elements (9,10).

While physical inactivity clearly has a negative impact on health favoring an inflamed environment, regular, moderate exercise is beneficial against systemic inflammation and counteracts the development of chronic diseases (11). Besides prevention, exercise also is an effective therapeutic strategy to treat obesity, type 2 diabetes, sarcopenia and neurodegeneration (12-14).

At the molecular level, many of the beneficial effects of exercise are mediated by the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (15). PGC-1α is transiently induced by a single bout of exercise and chronically elevated in endurance trained muscle (16). Activated PGC-1α then controls the expression of genes encoding proteins involved in mitochondrial biogenesis, oxidative phosphorylation and other features of oxidative muscle fibers (17). Accordingly, mice with transgenic skeletal muscle-specific PGC-1α overexpression perform better in endurance tests and display a switch towards oxidative type I and type IIA fibers (18,19). The increased fitness of healthy transgenic animals translates into improvement of symptoms when PGC-1α is expressed in the context of different muscle wasting conditions as shown for Duchenne muscular dystrophy, sarcopenia, a mitochondrial myopathy and denervation- or lovastatin-induced fiber atrophy (20-22).

Inversely, a skeletal muscle-specific deletion of the PGC-1α gene facilitates a type IIB and type IIX fiber type switch, reduces exercise performance and promotes muscle fiber damage (23). Furthermore, loss of PGC-1α results in elevated levels of pro-inflammatory factors locally in muscle as well as systemically (24). These findings suggest an anti-inflammatory role for PGC-1α: in fact, in skeletal muscle of diabetic patients, PGC-1α levels negatively correlate with IL-6 or TNFα levels independent of BMI (24).

PGC-1β, a close related member of the PGC-1 gene family, also exhibits dysregulated expression in skeletal muscle of diabetic patients and thereby contributes to the mitochondrial dysfunction observed in type 2 diabetes (25). While both PGC-1s share the ability to boost oxidative metabolism, PGC-1β is not regulated by exercise and primarily drives the formation of type IIX fibers (26). Interestingly, PGC-1β is required for alternative activation of and mitochondrial ROS production in macrophages (27,28); an immunomodulatory role in skeletal muscle has however not been described so far.

Based on these observations, we now tested the hypothesis that the PGC-1 coactivators exert anti-inflammatory effects in muscle. More precisely, we explored if PGC-1α and PGC-1β are able to modify cytokine expression upon exposure of muscle cells to different inflammatory stimuli like TNFα, TLR agonists and free fatty acids (FFAs). We found that the PGC-1s repress the transcriptional activity of p65 and thereby modulate the NF-κB signaling pathway. These data represent a prime example of crosstalk between metabolic and immune pathways with important implications for skeletal muscle function.

## 2.3 Materials and Methods

### 2.3.1 Cell culture and treatments

The mouse skeletal muscle cell line C2C12 was maintained below confluence in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum and 1x Penicillin/Streptomycin (Invitrogen). For differentiation into myotubes, growth medium was exchanged for DMEM supplemented with 2% horse serum (Invitrogen) for at least 3 days. PGC-1α, PGC-1β and GFP were overexpressed from recombinant adenoviral constructs 48 h prior to treatment. Stimulation with TNFα (Sigma-Aldrich) and TLR agonists (Invivogen) in growth or differentiation medium lasted for 2 h unless otherwise stated. Concentrations were as follows: TNFα 10ng/ml, PAM3CSK4 1μg/ml (TLR1/2 agonist), HKLM $10^8$ cells/ml (TLR2 agonist), poly(I:C) 25μg/ml (TLR3 agonist), *E.coli* K12 LPS 1μg/ml (TLR4 agonist), *S. typhimurium* flagellin 1μg/ml (TLR5 agonist), FSL1 1μg/ml (TLR6/2 agonist), ssRNA40 1μg/ml (TLR8 agonist) and ODN18266 5μM (TLR9 agonist). Free fatty acids (FFA, Sigma-Aldrich) were dissolved in ethanol and further diluted to 1mM final concentration in DMEM containing 2% fatty acid- and endotoxin-free bovine serum albumin (Sigma-Aldrich); FFA treatment lasted for 16 h in serum-free medium. The protein phosphatase inhibitor okadaic acid (OA, Sigma-Aldrich, 250nM) was present 30 minutes prior to and during treatment with TNFα where indicated, while control samples were incubated with vehicle (DMSO, 0.04%) alone for equal times. The PPARα inhibitor MK 886 (Tocris Bioscience, 20μM) was present 24 h prior to and during treatment with TNFα where indicated, while control samples were incubated with vehicle (DMSO, 0.02%) alone for equal times.

### 2.3.2 Semiquantitative real-time PCR

RNA was isolated from treated C2C12 cells using Trizol (Invitrogen) and residual DNA contamination was removed by DNase I digestion (Invitrogen). 1μg of RNA was reverse transcribed with SuperScript II (Invitrogen) and the resulting cDNA used as template for RT-PCR. To detect relative expression levels, cDNA was amplified with the SYBR Green Master mix (Applied Biosystems) and analyzed on a StepOnePlus RT-PCR System (Applied Biosystems). The respective primer pairs are listed in Suppl. Table 1. All values are normalized to the expression of TATA-Box binding protein (TBP) and expressed as fold induction over the untreated control condition.

### 2.3.3 ELISA

To determine cytokine concentrations in cell culture supernatants, sandwich immunoassays against IL-6 were performed according to the manufacturer's instructions (Quantikine, R&D Systems).

### 2.3.4 NF-κB Customized Array analysis

Differentiated C2C12 cells overexpressing PGC-1α, PGC-1β or GFP were treated with TNFα for 2h and RNA was extracted (NucleoSpin RNA II, Macherey-Nagel). The custom array analysis was subsequently performed as previously described (29). Briefly, RNA was processed with the Amino Allyl MessageAMP II aRNA Amplification Kit (Ambion) according to the manufacturer's instructions to yield aminoallyl-modified aRNA that was further coupled to Cy3 or Cy5 dyes respectively. Biological duplicates were labeled crosswise to control for dye effects. Pairs of samples were hybridized on a total of 14 slides containing probes for 524 genes included in the array as described elsewhere (29, ArrayExpress A-MEXP-1502). Significantly regulated genes as determined by ANOVA ($P \leq 0.01$) were subject to cluster analysis. The 3 resulting clusters were the source of further KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) terms enrichment analysis with FatiGO (30) considering the adjusted p-value$\leq$0.05 as significant. To predict the relevant transcription factors for the regulation of each cluster, motif search within a region spanning +200bp to -800bp around the transcription start site (TSS) of each gene in the array was performed using MotEvo (31) in combination with a non-redundant set of position weight matrices mainly derived from JASPAR and TRANSFAC. Overrepresented motifs were retrieved by comparing binding site occurrence in each cluster to the occurrence in the whole array and considered significant with a z score $\geq$2. Microarray data were further analyzed with MARA (Motif Activity Response Analysis (32)) to predict the most important transcription factors that contribute to the changes in gene expression in our set of samples (independent of clustering).

### 2.3.5 Dual-luciferase reporter gene assays

90 – 95% confluent C2C12 myoblasts were transfected using Lipofectamin 2000 (Invitrogen) with plasmids containing firefly luciferase under the control of 3 NF-κB sites (wild-type, wt) or a construct with mutated sites as control in combination with renilla luciferase as internal control. Cells were then either treated with TNFα / TLR agonists for 2h, or co-transfected with p65, increasing

amounts of PGC-1α/β and empty vector (pcDNA3.1) to keep total DNA concentration constant. Cells were assayed 24h post transfection with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Briefly, cells were lysed and firefly and renilla luciferase activities determined on a Centro LB 960 luminometer (Berthold Technologies). Firefly-derived luciferase values were normalized to renilla activity and results expressed as ratio of wt to mutated reporter with the control condition being arbitrarily set to 1.

### 2.3.6 TransAM NF-κB DNA binding assays

Nuclear extracts from myotubes were prepared by swelling of the cells in hypotonic buffer (20mM Hepes, pH 7.5, 5mM NaF, 10μM $Na_2MoO_4$, 0.1mM EDTA) and breaking the cytoplasmic membrane with Nonidet P-40 substitute (0.5%). After centrifugation, the residual nuclear pellet was resuspended in Complete Lysis Buffer to dismantle nuclear membrane integrity and obtain the nuclear extract as supernatant of the final, fast centrifugation step. To determine the amount of active NF-κB in these nuclear extracts, TransAM assays (Active Motif) were performed following the manufacturer's instructions. In brief, 10μg of nuclear extracts were added to wells coated with oligonucleotides containing the NF-κB consensus sequence (5'-GGGACTTTCC-3'). These oligonucleotides trap active transcription factors, which then are detected in an ELISA-like assay with specific primary antibodies for the different NF-κB subunits and a secondary horseradish peroxidase (HRP)-conjugated antibody. A colorimetric reaction accordingly determines the amount of NFκB-DNA binding. Results are expressed in arbitrary units minus the respective blank value, and normalized to Ctrl conditions.

### 2.3.7 Western Blotting

After washing away residual culture medium with PBS, cells were scraped off the dish in lysis buffer (20mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM $Na_4P_2O_7$,1mM β-glycerophosphate, 1mM $Na_3VO_4$) supplemented with protease (complete tablets, Roche) and phosphatase inhibitors (phosphatase inhibitor cocktail 2, Sigma-Aldrich) and kept on ice for 10 min with occasional vortexing. Centrifugation at 10'000g yielded the protein lysate, which was subjected to SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane (Whatman) on a semi-dry electroblotting system (Thermo Scientific) with 1.5mA/cm$^2$ and transfer confirmed by Ponceau S staining (0.1% in 5% acetic acid). Proteins were detected with the following

primary antibodies: anti-p65, anti-Pp65(ser536) (both Cell Signaling Technology), anti-p105/p50 (abcam), anti-p100/p52, anti-RelB, anti-cRel, anti-IκBα, anti-IκBβ, anti-IKKα, anti-IKKγ, anti-α-tubulin as loading control (all Cell Signaling Technology) and an anti-rabbit-IgG HRP-coupled secondary antibody (Dako). Bands were finally visualized by autoradiography with ECL substrate (Pierce) and density quantified with ImageJ software.

### 2.3.8 Statistical analysis

Data were analyzed with student's *t* test using $P \leq 0.05$ as the significance threshold.

## 2.4 Results

### 2.4.1 PGC-1α and PGC-β differentially suppress pro-inflammatory cytokine expression and secretion

To delineate the effect of PGC-1s on inflammatory processes in muscle, myotubes overexpressing either adenovirally encoded PGC-1α or PGC-1β were compared to GFP-expressing control cells treated with different inflammatory stimuli. PGC-1 coactivator overexpression was high and functional as evident from the induction of the known target gene medium-chain acyl-coenzyme A dehydrogenase (MCAD, Suppl. Fig. 1A and 1B). Ectopic, recombinant TNFα strongly induced the gene expression of the pro-inflammatory markers IL-6, TNFα and macrophage inflammatory protein-1α (MIP-1α/CCL3). While PGC-1α did not negatively affect basal levels of these pro-inflammatory cytokines, it diminished their induction by TNFα yielding lower levels than in control cells (Fig. 1A). PGC-1β suppressed both basal and TNFα-induced expression of IL-6, TNFα and MIP-1α (Fig. 1B). As a consequence of the PGC-1-mediated repression in gene expression, lower levels of secreted IL-6 protein were observed in culture media after TNFα treatment (Fig. 1C and 1D).

Next, we examined the effect of PGC-1s on TLR stimulation in muscle cells. First, we assessed the expression pattern of the TLRs in our experimental system and found that TLR1, TLR2, TLR3, TLR 4, TLR6, and at lower levels TLR5 were detectable in the muscle cells (Fig. 2A). To study the activity of the TLRs, selective agonists for TLR2, TLR3, TLR4, TLR5, TLR8, TLR9, or the TLR1/2 and TLR6/2 heterodimers were applied. Of those, only TLR1/2, TLR4 and TLR6/2 activators consistently elevated expression of IL-6 and TNFα (Fig. 2B). Moreover, the same compounds increased luciferase reporter gene activity controlled by 3 repeats of a minimal NF-κB DNA-response element (Fig. 2C). For the subsequent experiments, agonists for TLR1/2, TLR4 and TLR6/2, the active TLRs in muscle cells, were utilized and compared to the TLR3 activator that was used as a negative, internal control. TLR1/2, TLR4 and TLR6/2 agonists all induced expression of IL-6 and TNFα in control cells as expected (Fig. 2D). Interestingly, PGC-1α effectively repressed TLR-mediated TNFα expression by all of the active agonists while IL-6 expression was only reduced by PGC-1α in the TLR1/2 and TLR4 agonist treatment (Fig. 2D). PGC-1α furthermore lowered basal IL-6 protein secretion, but not the elevated IL-6 secretion caused by TLR agonist stimulation (Fig. 2E). In contrast, PGC-1β significantly decreased IL-6 and TNFα gene expression both in the basal state and after activation of TLR1/2 while no effect was observed in the TLR4 or TLR6/2 agonist treated cells (Fig. 2F). Nevertheless however, IL-6 secretion into culture medium was strongly repressed by PGC-1β in all of the different experimental conditions (Fig. 2G).

As a third inflammatory stimulus besides TNFα and TLR agonists, the free fatty acids (FFA) palmitic acid ($C_{16}H_{32}O_2$), oleic acid ($C_{18}H_{34}O_2$), myristic acid ($C_{14}H_{28}O_2$), stearic acid ($C_{18}H_{36}O_2$), linoleic acid ($C_{18}H_{32}O_2$) and elaidic acid ($C_{18}H_{34}O_2$) were administered to muscle cells to mimick the lipid overload that is associated with disease progression in the metabolic syndrome. As expected, saturated fatty acids, in particular stearic and palmitic acid and to a lesser extent the shorter-chain species, produced a strong pro-inflammatory response as indicated by the transcriptional induction of

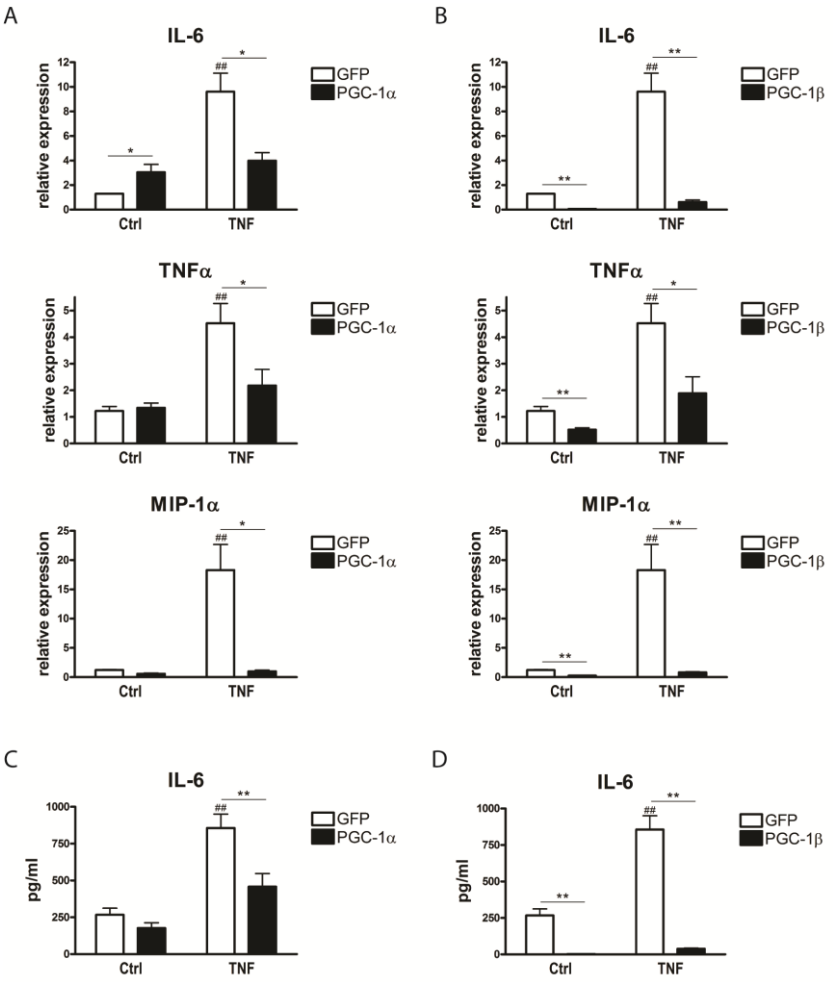


**Figure 1** ***PGC-1α and PGC-1β suppress TNFα-induced pro-inflammatory cytokines. A-D***, Differentiated C2C12 myotubes overexpressing PGC-1α and GFP (Panels A and C) or PGC-1β and GFP (Panels B and D) were treated with TNFα for 2h. Expression of pro-inflammatory cytokines was determined by real-time PCR (Panels A and B) and release of IL-6 into the medium quantified by ELISA (Panels C and D). Values represent the mean of at least 3 independent experiments +SEM. ## $P \leq 0.01$ GFP TNF versus GFP Ctrl, * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1α/β versus GFP.

the IL-6, TNFα and MIP-1α genes (Fig. 3A). In contrast, mono- or polyunsaturated fatty acids did not alter pro-inflammatory cytokine expression (Fig. 3A); in fact, oleic acid was even able to reverse the negative effects of palmitic acid (Fig. 3A) as previously described (33). Strikingly, PGC-1α potently inhibited the increase in IL-6, TNFα and MIP-1α gene expression mediated by palmitic, myristic and stearic acid (Fig 3A). Likewise, PGC-1β also efficiently blocked the FFA-induced elevation of IL-6

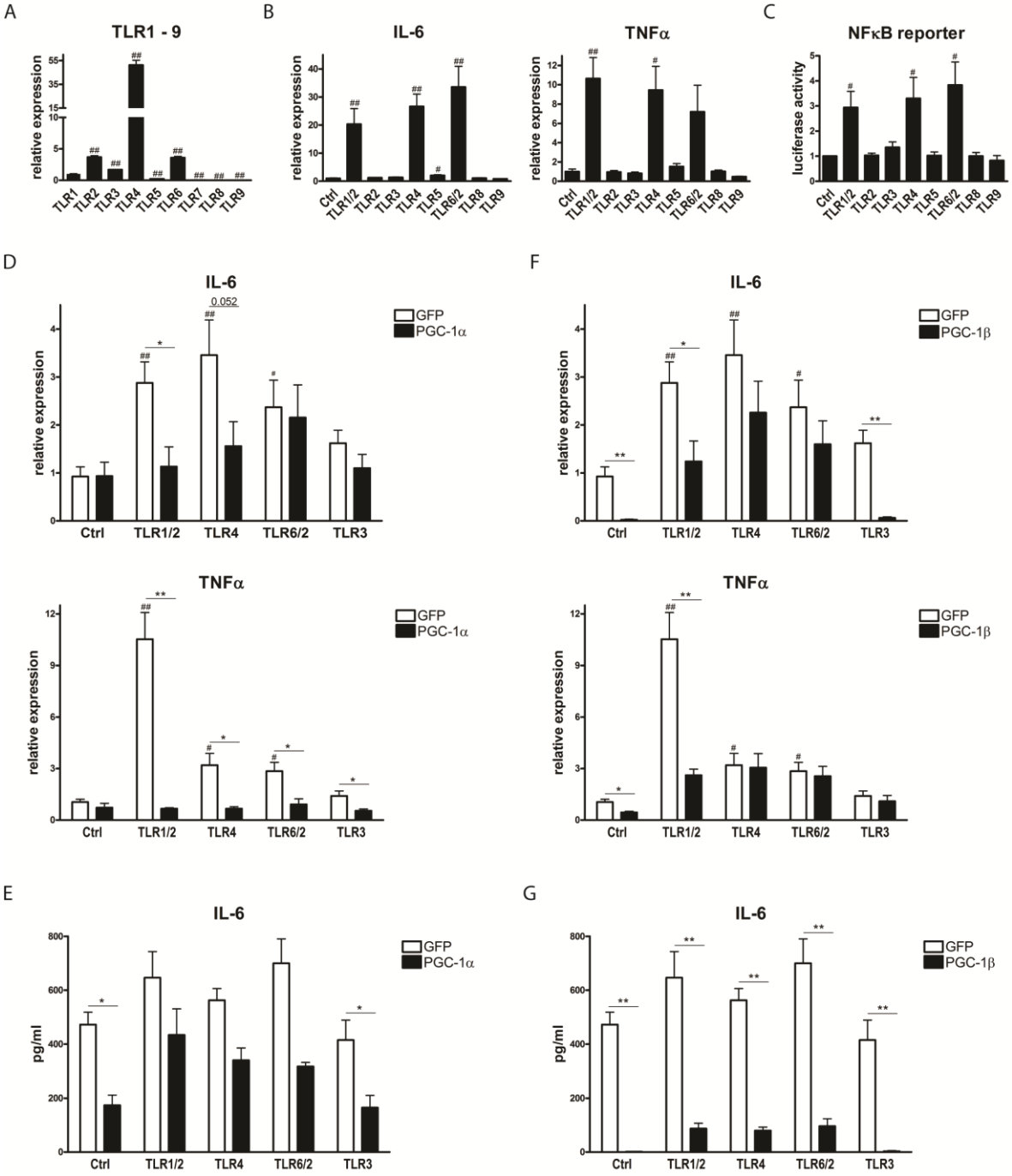


**Figure 2** ***PGC-1α and PGC-1β differentially suppress TLR agonist-induced pro-inflammatory cytokines.*** **A**, Differentiated C2C12 myotubes were tested for mRNA expression of TLR1-9. Expression values are arbitrarily normalized to those of TLR1. **B**, C2C12 cells were differentiated and treated with different TLR agonists for 2h. Expression of IL-6 and TNFα was assessed by real-time PCR. **C**, C2C12 cells were transfected with an NF−κB reporter construct (or a mutated reporter construct as control) and treated with different TLR agonists for 2h. Luciferase activity was determined and is expressed as ratio of wt to mutated reporter gene values. **D-G**, Differentiated C2C12 myotubes overexpressing PGC-1α and GFP (Panels D and E) or PGC-1β and GFP (Panels F and G) were treated with agonists for TLR1/2, TLR4, TLR6/2 and TLR3 for 2h. Expression of pro-inflammatory cytokines was determined by real-time PCR (Panels D and F) and release of IL-6 into the medium quantified by ELISA (Panels E and G). Values represent the mean of at least 3 independent experiments +SEM. # P≤0.05, ## P≤0.01 TLR agonist treated versus Ctrl (A, B, D-G) or TLR expression versus TLR1 (C), * P≤0.05, ** P≤0.01 PGC-1α/β versus GFP.

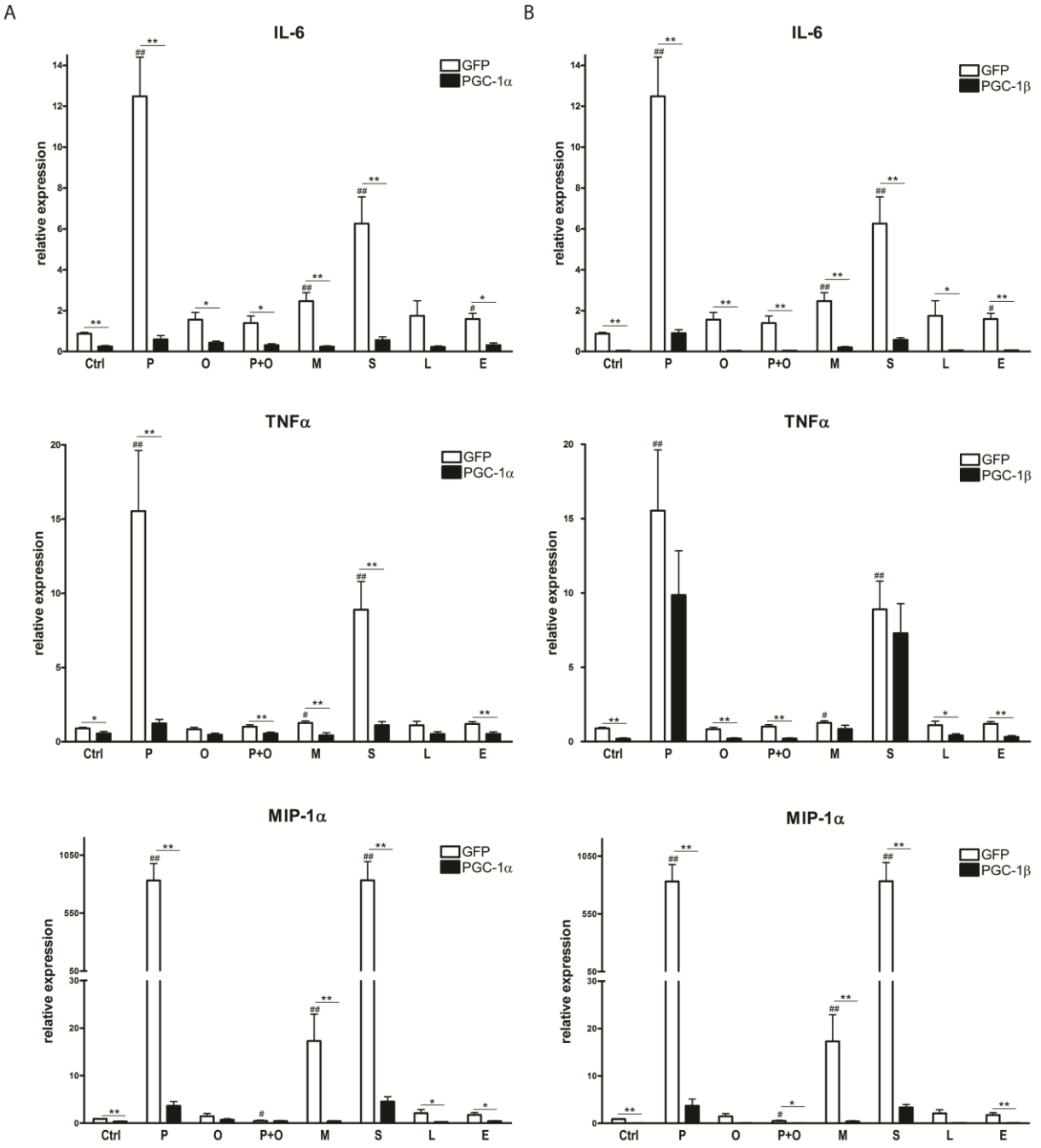


**Figure 3** ***PGC-1α and PGC-1β differentially suppress fatty acid-induced pro-inflammatory cytokines. A***, ***B***, Differentiated C2C12 myotubes overexpressing PGC-1α and GFP (Panel A), or PGC-1β and GFP (Panel B) were treated with different fatty acids (P=palmitic acid, O=oleic acid, M=myristic acid, S=stearic acid, L=linoleic acid, E=elaidic acid) for 16h. Expression of pro-inflammatory cytokines was determined by real-time PCR. Values represent the mean of at least 3 independent experiments +SEM. # P≤0.05, ## P≤0.01 GFP FFA versus GFP Ctrl, * P≤0.05, ** P≤0.01 PGC-1α/β versus GFP.

and MIP-1α transcript levels (Fig. 3B). Interestingly however, transcriptional elevation of TNFα by palmitic, myristic and stearic acid was unaffected by overexpression of PGC-1β (Fig 3B).

### 2.4.2 PGC-1α and PGC-1β target the NF-κB pathway to suppress inflammation

To examine the mechanisms behind the repressive effect of the PGC-1 coactivators on TNFα-induced pro-inflammatory cytokines, a customized array designed to represent the most important inflammatory genes and other NF-κB targets (29) was employed. Out of the 524 genes that are present on the array, 55 genes were found to be differentially regulated by TNFα treatment and/or PGC-1 overexpression ($P$ <0.01) and thus further analyzed. These 55 genes were grouped into 3 clusters (Fig. 4A): cluster 1 contained 21 genes that were upregulated by PGC-1α and PGC-1β including vascular endothelial growth factor (VEGF), a known PGC-1α target (34). The 13 and 21 genes in clusters 2 and 3, respectively, were repressed by PGC-1α and PGC-1β. Importantly, genes in cluster 3 were TNFα-inducible while the expression of the genes in cluster 2 was not modulated by TNFα treatment (Fig. 4A). To validate the results of the microarray, three representative genes from each cluster were chosen and their expression analyzed by real-time PCR. Mitochondrial translational initiation factor 2 (Mtif2), VEGFα and protein arginine methyltransferase 1 (Prmt1) from cluster 1 were indeed induced (Suppl. Fig. 2A) while chemokine (C-X-C motif) ligand 12 (Cxcl12), complement component 2 (C2) and E2F transcription factor 2 (E2f2) from cluster 2 (Suppl. Fig 2B) and chemokine (C-C motif) ligand 2 (Ccl2), Ccl7 and Cxcl1 from cluster 3 (Suppl. Fig. 2C) were repressed by PGC-1 coactivators confirming the results of the microarray. Furthermore, TNFα-inducibility of genes in cluster 3 was verified.

Functionally, the genes in cluster 1 were enriched in only one Gene Ontology (GO) term compared to 18 significant terms in cluster 2 and 96 terms in cluster 3 (of which only 46 terms with a p-value≤0.01 are shown) (Suppl. Fig. 3). 12 of the GO categories in cluster 2 and 26 categories in cluster 3 are related to inflammation and immunity. Importantly, the inflammation-related GO terms comprise the top 10 and top 15 ranking categories in clusters 2 and 3, respectively (Suppl. Fig. 3). Likewise, all 3 KEGG pathways assigned to cluster 2 and the top 9 KEGG pathways out of 11 of cluster 3 are related to inflammatory signaling (Fig. 4B). These results suggest that the PGC-1s are able to repress inflammatory processes in muscle cells.

To predict which transcription factors are involved in the regulation of each cluster, promoter regions were analyzed in regard to their motif composition. Motifs overrepresented in each of the clusters are listed in Suppl. Table 2A. As background for this analysis, all promoters of the microarray were used, thereby eliminating any putative bias that might have been introduced with the specific choice of genes in the customized microarray. The majority of predicted binding sites were unique to one cluster, i.e. 26 out of 33 motifs in cluster 1, 20 out of 26 motifs in cluster 2 and 21 out of 28 motifs in cluster 3 (Suppl. Table 2B). Intriguingly, one distinct transcription factor binding motif stood out as the clear top ranking candidate in each of the three clusters based on z score, namely NFY{A,B,C} in cluster 1 (z score of 9.74 compared to the second ranking z score of 4.9 for GFI1), IRF1,2,7 in cluster

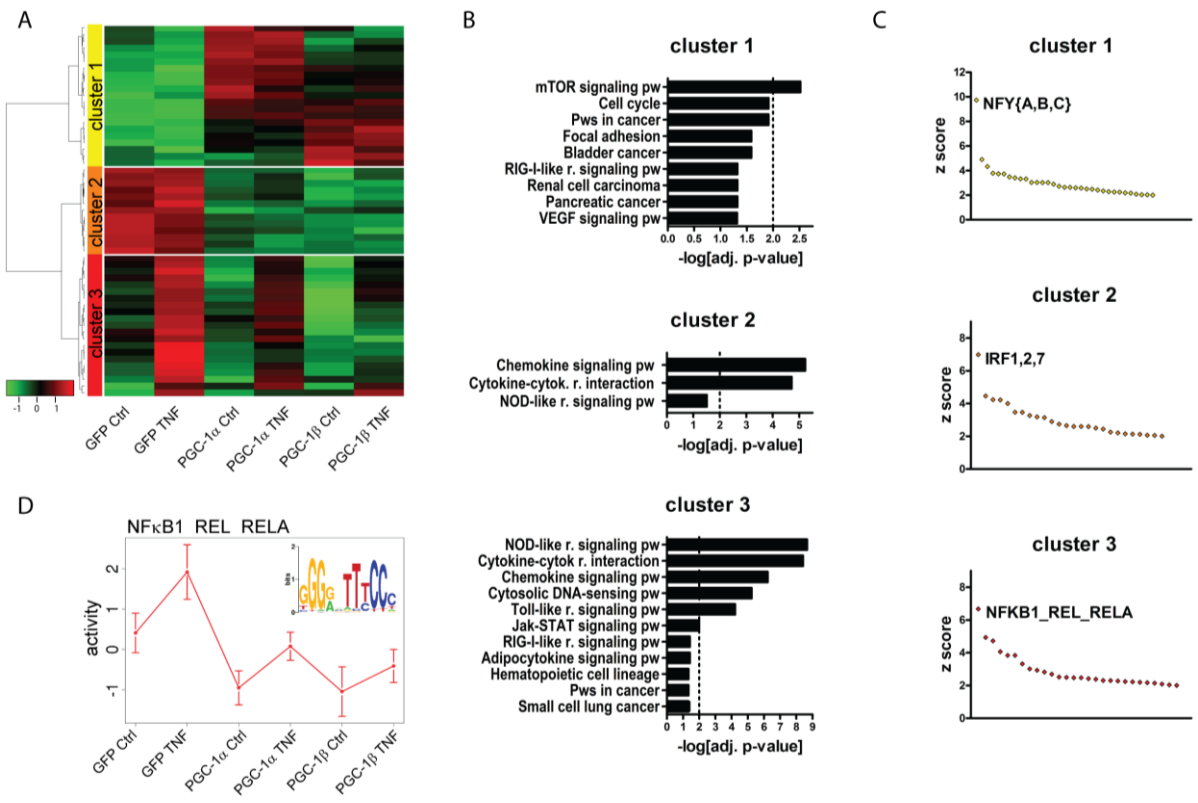


**Figure 4** ***Inflammatory pathways and NF-κB activity are suppressed by PGC-1s.*** Differentiated C2C12 myotubes overexpressing PGC-1α, PGC-1β and GFP were treated with TNFα for 2h and subsequently subjected to a customized microarray analysis. **A**, Significantly regulated genes ($P \leq 0.01$) were clustered and are depicted as heat map. **B**, KEGG pathways enriched within each cluster (adjusted p-value≤0.05) are shown, the dotted line indicates an adjusted p-value of 0.01. (Abbreviations: cytok. = cytokine, pw = pathway, r. = receptor). **C**, Motifs overrepresented in the promoters of each cluster were identified and their z score distribution plotted. For a complete list see **Suppl. Table 2A. D,** Activity plot of NF-κB (top scoring transcription factor motif) over different conditions as predicted by MARA (Motif Activity Response Analysis) and the corresponding sequence logo of the position weight matrix.

2 (z score of 6.98 compared to 4.46 for FOX{F1,F2,J1}) and NFκB1_REL_RELA in cluster 3 (z score of 6.67 compared to 4.93 for AR) (Fig. 4C and Suppl. Table 2A). These predictions indicate that the NF-κB pathway is a likely target for the PGC-1s to suppress TNFα-inducible inflammatory gene expression represented in cluster 3. Accordingly, NF-κB was also the highest scoring motif in the Motif Activity Response Analysis (MARA) of the whole array and thus the most likely transcription factor to modulate TNFα- and PGC-1-dependent gene expression (Fig. 4D).

### 2.4.3 PGC-1β reduces p65 and p50 expression levels

To experimentally validate the biocomputational prediction of PGC-1-mediated repression of NF-κB signaling as the central mechanism for the anti-inflammatory effect of the PGC-1 coactivators on TNFα-inducible genes, reporter gene assays were performed with a construct containing the luciferase gene under the control of 3 NF-κB DNA response elements. The activity of the reporter gene construct in response to TNFα treatment resembles endogenous IL-6 and TNFα gene expression (Suppl. Fig. 1C and 1D). Increasing amounts of co-transfected PGC-1α or PGC-1β progressively inhibited transcription from a NFκB-responsive promoter that is activated by exogenous p65 (Fig. 5A).

To elucidate the mechanism by which the PGC-1 coactivators repress NF-κB activity even on minimal NF-κB response element-driven gene expression, we first examined the expression levels of the different NF-κB family members before and after TNFα treatment and PGC-1 overexpression, respectively (Fig. 5B and 5C). TNFα led to a significant increase in transcript levels of all 5 NF-κB isoforms (p65, p105/p50, p100/p52, RelB and c-Rel) in GFP-infected control cells. PGC-1α did not change the levels of the canonical isoforms p65 and p105/p50 as well as RelB in the basal, vehicle-treated cells while an induction of p100/p52 was observed suggesting a switch towards the noncanonical NF-κB pathway. In contrast to the first 4 NF-κB isoforms, c-Rel expression was clearly reduced by PGC-1α overexpression (Fig. 5B). After TNFα treatment, the levels of all NF-κB isoforms tended to be lower in PGC-1α overexpressing cells, however only reaching statistical significance in the case of c-Rel gene expression (Fig 5B). In striking contrast to the PGC-1α-mediated effect, PGC-1β suppressed p65 and p105/p50 as well as RelB and c-Rel gene expression in the basal state (Fig. 5C). The transcript levels of all 5 NF-κB isoforms tended to be lower in PGC-1β overexpressing cells after TNFα treatment; similar to PGC-1α overexpressing cells, this repression was however only significant for c-Rel gene expression (Fig. 5C). Therefore, while the basal repression of p65 and p105/p50 could contribute to the strong repression of inflammatory gene expression by PGC-1β in non-stimulated cells, it is unlikely that the small differences in expression levels of the different NF-κB isoforms after TNFα treatment underlie the profound effects of PGC-1s on pro-inflammatory cytokine expression in stimulated muscle cells. Thus, to further elucidate the molecular mechanism underlying this observation, we next assessed the DNA binding capability of all NF-κB family members in the context of TNFα treatment and PGC-1 overexpression (Fig. 5D and 5E). Of the 5 NF-κB isoforms, RelB and c-Rel were undetectable in nuclear extracts from C2C12 myotubes in the TransAM assay and are thus unlikely to play a major role in the regulation of pro-inflammatory cytokine expression in our experimental context of muscle cells as hypothesized in other publications (35). The DNA binding of p50 and p52 was detectable though low (Fig. 5D and 5E). In contrast, recruitment of p65 to DNA response elements was substantial in non-stimulated cells and further

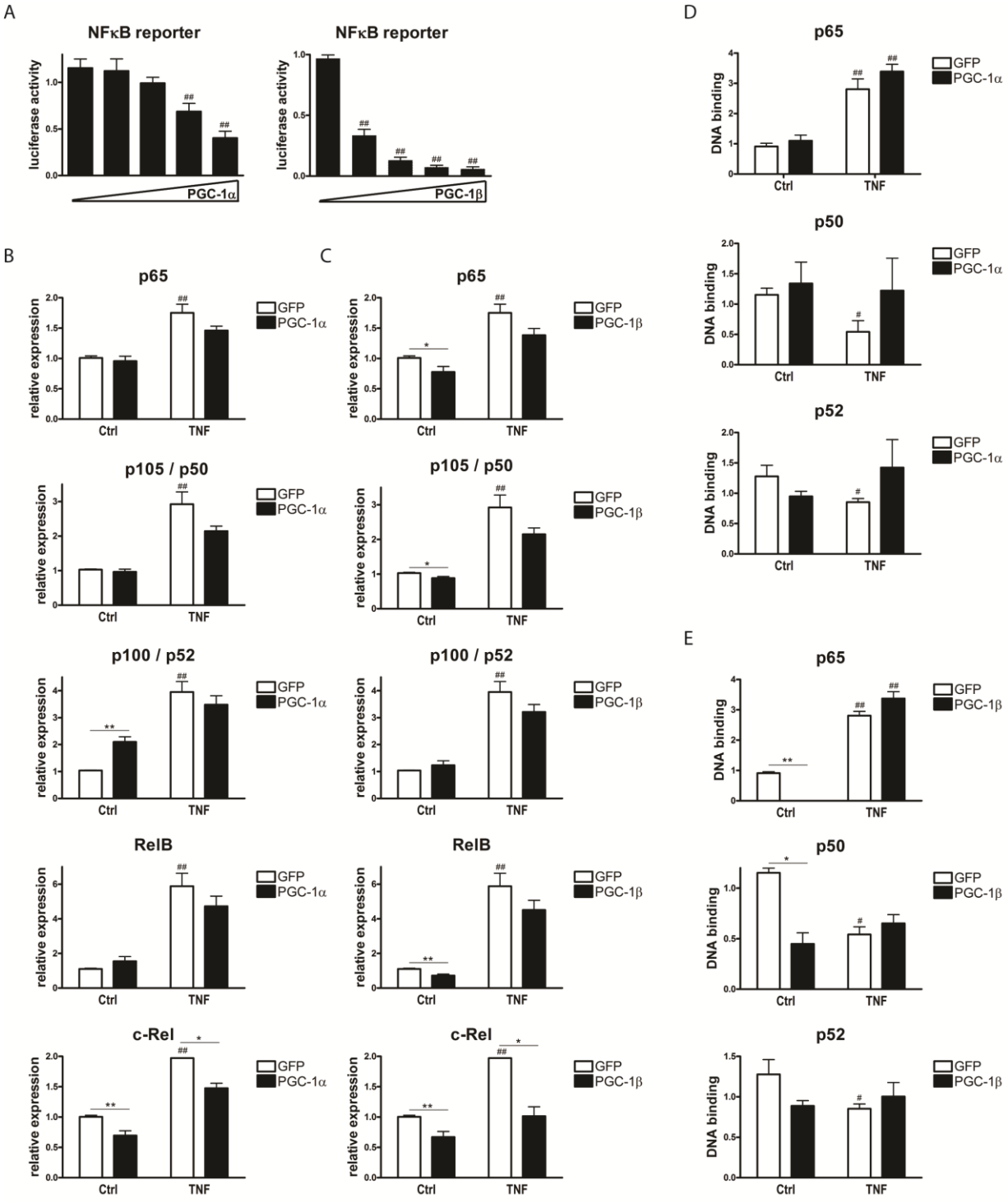


**Figure 5** ***PGC-1s suppress NF-κB transcription activation potential without changing DNA binding or affecting NF-κB expression levels.*** **A**, C2C12 cells were transfected with a wild-type and a mutated NF−κB reporter construct, p65 and increasing amounts of PGC-1α or PGC-1β. Luciferase activity was determined after 24h and is expressed as ratio of wt reporter to mutated reporter gene expression. ## $P< 0.01$ PGC-1 versus Ctrl. **B**-**E**, Differentiated C2C12 myotubes overexpressing PGC-1α and GFP (Panels B and D) or PGC-1β and GFP (Panels C and E) were treated with TNFα for 2h. **B**, **C**, Expression of NF-κB family members was determined by real-time PCR. **D**, **E**, DNA binding of NF−κB family members in nuclear extracts was measured by TransAM. Values represent the mean of at least 3 independent experiments +SEM. # $P\leq0.05$, ## $P\leq0.01$ GFP TNF versus GFP Ctrl, * $P\leq0.05$, ** $P\leq0.01$ PGC-1α/β versus GFP.

elevated after TNFα treatment. Interestingly however, DNA binding of p65 with and without TNFα treatment, respectively, was not changed by PGC-1α (Fig. 5D) while PGC-1β strongly inhibited binding of p65 and p50 to DNA in the basal, vehicle-treated muscle cells (Fig. 5E). Comparable to PGC-1α overexpression, DNA binding of any of the NF-κB isoforms was not affected by ectopic PGC-1β in TNFα-stimulated cells (Fig. 5E). It is thus conceivable that both low p65/p50 expression as well as reduced DNA binding account for the PGC-1β-mediated reduction in pro-inflammatory cytokine expression compared to vehicle-treated control cells. In contrast however, the diminished levels of these cytokines upon TNFα treatment in both PGC-1α- and PGC-1β-overexpressing cells can neither be attributed to changes in NF-κB expression nor to modulation of the NF-κB protein binding capability to DNA response elements.

### 2.4.4 PGC-1α and PGC-1β diminish the transcriptional activity of p65

Based on our data implying alternative molecular mechanisms distinct from transcriptional regulation or DNA binding of NF-κB to underlie the repressive action of the PGC-1 coactivators on the activity of this transcription factor, we next studied upstream signaling and the post-translational modification of p65 that influence the transcriptional activity of NF-κB. First, we examined the possibility that high levels of IκBs after TNFα treatment could account for lowered cytokine

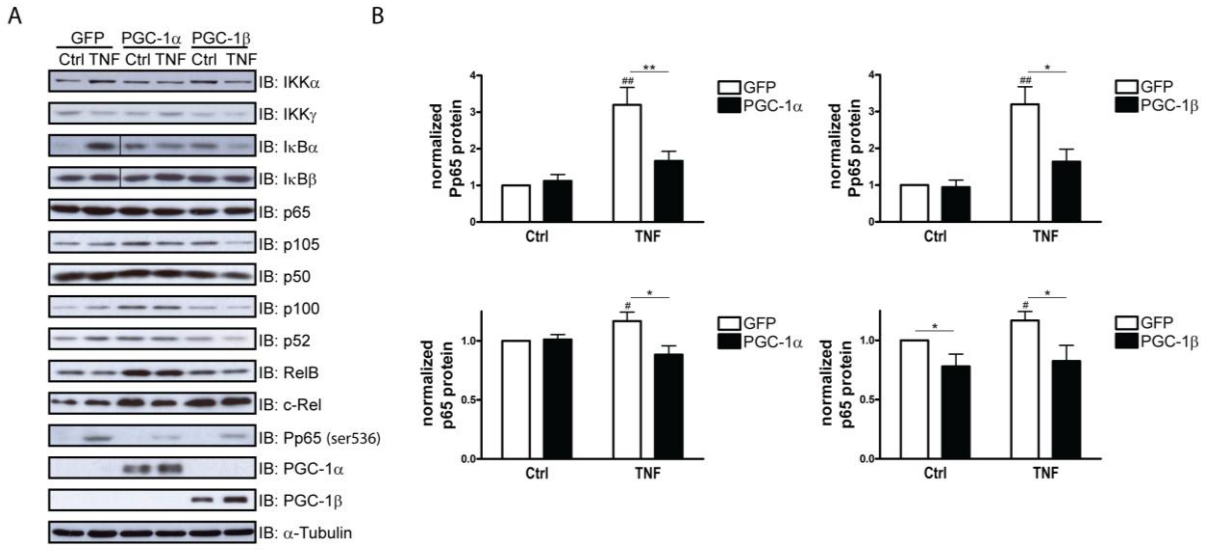


**Figure 6** ***PGC-1s diminish p65 phosphorylation at serine 536.*** Differentiated C2C12 myotubes overexpressing PGC-1α, PGC-1β and GFP were treated with TNFα for 2h. **A**, Protein abundance of NF−κB family members, pathway components, phospho-p65 (ser536), PGC-1α, PGC-1β and α-Tubulin was assessed by immunoblotting. **B**, Quantification of phospho-p65 (ser536) and total p65 protein levels, normalized to α-Tubulin. Values represent the mean of at least 3 independent experiments +SEM. # $P \leq 0.05$, ## $P \leq 0.01$ GFP TNF versus GFP Ctrl, * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1α/β versus GFP.

expression. However, the relative amount of IκBβ protein was not different between conditions (Fig. 6A). IκBα, an NF-κB target gene, was accordingly increased on the protein level by TNFα treatment and reduced in muscle cells overexpressing PGC-1α or PGC-1β (Fig. 6A and Suppl. Fig. 4A and 4B), similar to other NF-κB targets such as IL-6 and TNFα (Fig. 1) excluding the possibility of IκB-mediated repression. Subsequently, IKK protein levels were assessed: IKKβ was not and IKKγ was barely detectable (Fig. 6A). In contrast, IKKα was slightly increased by TNFα treatment; this effect was abrogated in cells overexpressing PGC-1α or PGC-1β (Fig. 6A). IKKα is one of the protein kinases that is able to phosphorylate p65 at serine 536. Importantly, the phosphorylation status of p65 at serine 536 affects the transcriptional activity of NF-κB even when bound to DNA response elements (10). In Western blot analyses of total and phosphorylated p65 protein, a small but significant increase in total p65 protein levels was observed after TNFα treatment in control cells (Fig. 6A and 6B). PGC-1α overexpression did not affect basal levels of p65, while PGC-1β diminished total p65 protein expression in this context, as expected based on the reduced mRNA expression of p65 in PGC-1β overexpressing muscle cells (Fig. 5C). Strikingly however, both PGC-1α and PGC-1β reduced TNFα-mediated phosphorylation of p65 by about 50% (Fig. 6A and 6B). None of the other NF-κB family members underwent regulation by TNFα on the protein level. Interestingly, PGC-1α overexpression resulted in elevated protein levels of p105, p100, p52 and RelB in non-stimulated muscle cells and, to a smaller extent, of p105 and p100 in TNFα-treated cells again suggesting a PGC-1α-dependent increase in the non-canonical NF-κB pathway (Fig. 6A and Suppl. Fig. 4A). In contrast, PGC-1β does not seem to elevate the non-canonical NF-κB family members like PGC-1α. Thus, besides the strong increase in c-Rel protein and the more moderate elevation of p105 protein in non-stimulated cells, PGC-1β significantly reduced RelB protein levels (Fig. 6A and Suppl. Fig. 4B). The discrepancy between the PGC-1-mediated repression of c-Rel gene expression compared to the elevation of c-Rel protein levels implies post-transcriptional effects in the regulation of this particular NF-κB family member.

### 2.4.5 Dephosphorylation and transrepression of p65 are potential molecular mechanisms for diminished cytokine expression

To further substantiate the findings implying that the PGC-1 coactivators modulate NF-κB activity by preventing p65 phosphorylation, we examined other upstream effectors for this phosphorylation as well as downstream events affecting the DNA-bound NF-κB transcriptional complex. The protein kinase Akt has been implicated in the regulation of the NF-κB signaling pathway upstream of IKKα (36), one of the protein kinases to mediate p65 phosphorylation and of which protein levels are

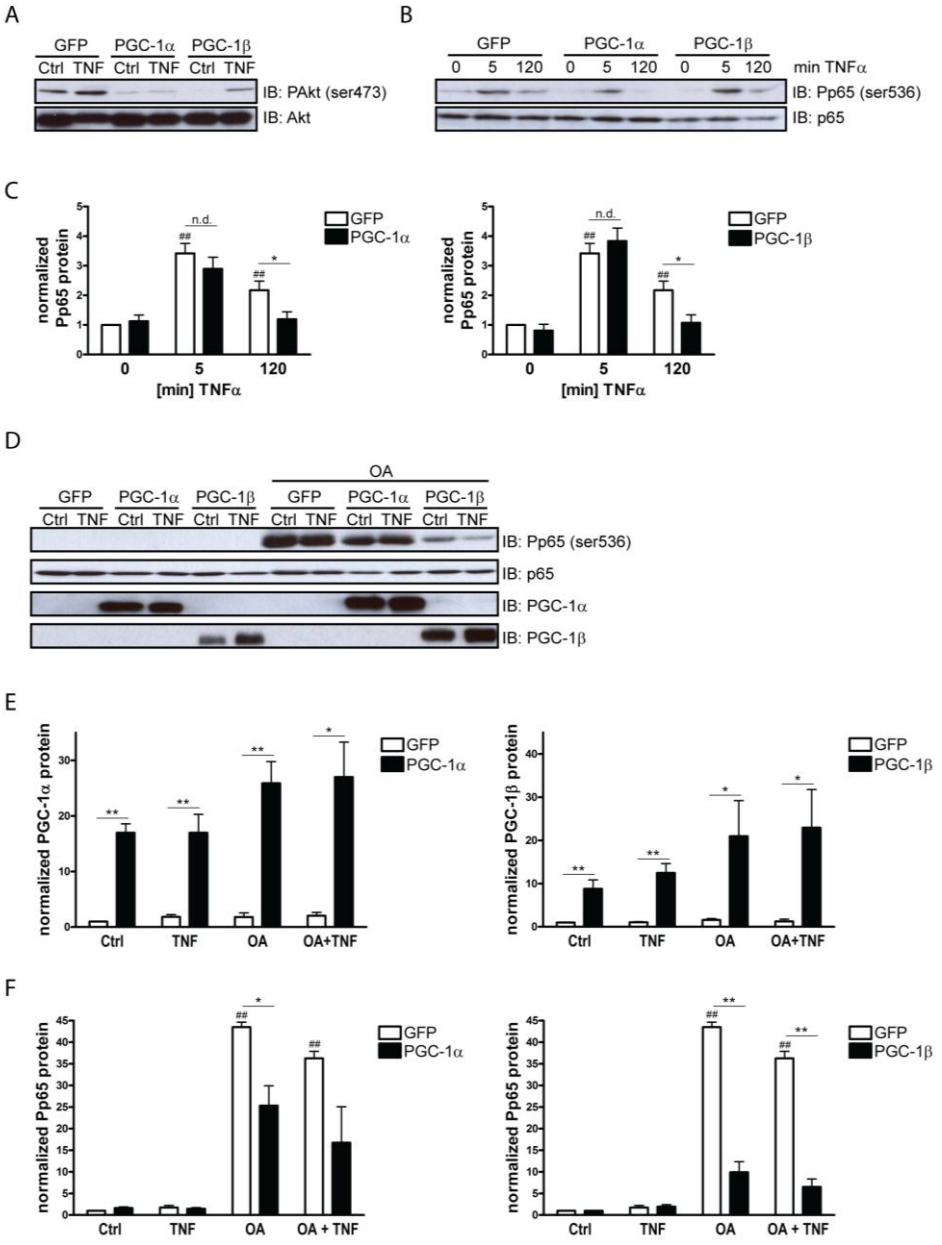


**Figure 7** ***Dephosphorylation of p65 is a potential molecular mechanism for diminished cytokine expression.*** **A,** Differentiated C2C12 myotubes overexpressing PGC-1α, PGC-1β and GFP were treated with TNFα for 2h. Protein levels of Akt and phospho-Akt (ser473) were determined by immunoblotting. **B,** Differentiated C2C12 myotubes overexpressing PGC-1α, PGC-1β and GFP were treated with TNFα for 5min and 2h, respectively. Protein abundance of Phospho-p65 (ser536) and total p65 was assessed by immunoblotting. **C,** Quantification of Phospho-p65 (ser536) levels from B, normalized to α-Tubulin. **D**, Differentiated C2C12 myotubes overexpressing PGC-1α, PGC-1β and GFP were treated with TNFα for 2h in the presence or absence of okadaic acid (OA). Protein abundance of phospho-p65 (ser536), total p65, PGC-1α and PGC-1β was assessed by immunoblotting **E, F,** Quantification of PGC-1α and PGC-1β protein levels from D, normalized to α-Tubulin (Panel E). Quantification of Phospho-p65 (ser536) protein levels from D, normalized to α-Tubulin (Panel F). Values represent the mean of at least 3 independent experiments +SEM. # $P \leq 0.05$, ## $P \leq 0.01$ GFP OA versus GFP Ctrl, * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1α/β versus GFP.

reduced by PGC-1α and PGC-1β overexpression (Fig. 6A). As expected, based on these findings, PGC-1α and PGC-1β diminished Akt activation as evident from diminished phospho-Akt (ser473) levels normalized to total Akt protein (Fig. 7A).

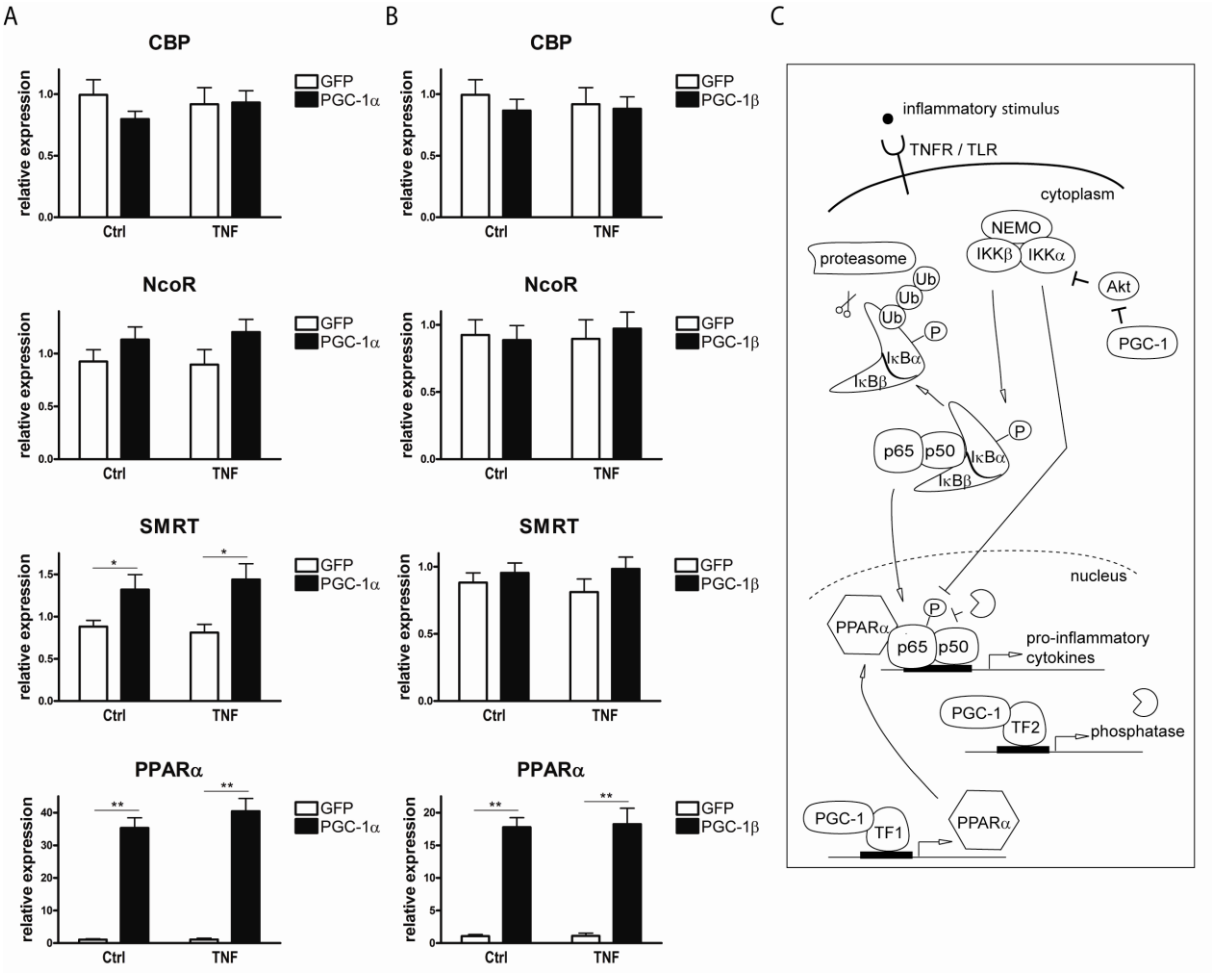


**Figure 8** ***Transrepression of p65 is a potential molecular mechanism for diminished cytokine expression.*** **A, B,** Differentiated C2C12 myotubes overexpressing PGC-1α and GFP (Panel A) or PGC-1β and GFP (Panel B) were treated with TNFα for 2h. Expression of coactivator and corepressor proteins, and of nuclear receptor PPARα was determined by real-time PCR. * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1α/β versus GFP. **C,** Proposed model of PGC-1α/β interference with NF−κB signaling. Gene-, activator- and PGC-1-specific repression of NF−κB target genes is mediated by reduced phosphorylation of p65, transcriptional induction of PPARα and subsequent transrepression as well as activation of an unknown protein phosphatase by both PGC-1s. Furthermore, PGC-1β specifically inhibits p65 and p50 expression while PGC-1α elevates members of the NF−κB family involved in the alternative, non-canonical activation (not depicted).

To obtain a more accurate picture of p65 phosphorylation, a time course experiment was performed. It revealed that p65 is phosphorylated at serine 536 after 5min of TNFα treatment even in the presence of PGC-1α and PGC-1β (Fig. 7B and 7C) implying the possibility that not only an altered kinase profile but also activity of a protein phosphatase might be involved in the PGC-1-mediated modulation of NF-κB phosphorylation. We therefore tested whether pharmacological inhibition of protein phosphatase 2A (PP2A) and PP1, two enzymes that dephosphorylate p65 (37), by

okadaic acid abolishes the repression of p65 phosphorylation mediated by PGC-1α and PGC-1β in muscle cells. As expected, okadaic acid powerfully stabilized phosphorylation of p65 at serine 536 (Fig. 7D and 7F). Strikingly however, PGC-1α and PGC-1β overexpression (Fig. 7D and 7E) still reduced p65 phosphorylation, even in okadaic acid-treated cells (Fig. 7D and 7F). Thus, while PP1 and PP2A clearly affect the serine 536 phosphorylation of p65 in our experimental context, these two phosphatases are most likely not involved in the modulation of p65 phosphorylation by the PGC-1s.

Since the phosphorylation status of p65 affects its affinity to cofactors (38), we also determined the expression levels of the coactivator CBP and corepressors NCoR and SMRT. Of those, only SMRT transcript levels were significantly increased by PGC-1α (Fig. 8A and 8B). A well-described mechanism of inhibition of inflammatory gene expression is mediated by nuclear receptors and termed transrepression (39). We evaluated the gene expression of potential candidates for transrepression and found PPARα to be strongly induced by both PGC-1α and PGC-1β in non-stimulated and TNFα-treated muscle cells similar to previous data (40) (Fig. 8A and 8B). PPARα therefore likely contributes to the PGC-1-dependent reduction in NF-κB transcriptional activity by impairing the exchange of corepressors for coactivators that is necessary to effectively initiate transcription. In fact, inhibition of PPARα recovered expression of IL-6 and TNFα in the presence PGC-1β (Suppl. Fig 5).

## 2.5 Discussion

With an aging population and an increasingly sedentary lifestyle, chronic diseases are on the rise. Obesity and its comorbidities but also some cancers and neurodegeneration have been associated with local and systemic inflammation that worsens disease progression, while exercise has beneficial effects in many of these disorders and even acts preventive (41). PGC-1α is a major molecular mediator of exercise in skeletal muscle and its loss not only disturbs metabolic processes but also evokes a local and systemic inflammation (24). In the present report we thus tested the idea that PGC-1 coactivators have anti-inflammatory properties.

Indeed, we confirmed such properties as PGC-1α and PGC-1β were able to diminish the increase in pro-inflammatory cytokines elicited by different inflammatory stimuli such as TNFα, TLR agonists and saturated FFAs. We identified the NF-κB pathway as main target of PGC-1-dependent repression. These results are complemented by *in vivo* findings of Brault and coworkers who showed that NF-κB reporter activity decreases after electroporation of tibialis anterior muscle with PGC-1α / PGC-1β in the context of anti-atrophic effects of PGC-1s (42). Similar conclusions were derived from experiments in human aortic smooth muscle and endothelial cells where PGC-1α suppressed TNFα-induced VCAM-1 and MCP-1 expression that contribute to inflammation in atherosclerosis (43).

Mechanistically, PGC-1α and PGC-1β lower phosphorylation of the NF-κB family member p65 which limits its transcriptional activation potential. This is further substantiated by data from muscle-specific PGC-1α transgenic animals that also exhibit reduced p65 phosphorylation (44). Diametrically opposed to this report, another recent publication states that p65 phosphorylation is higher in PGC-1α transgenic muscle before as well as after injection of TNFα (45). The exact role of PGC-1α on muscle inflammation *in vivo* thus remains unresolved. In our cellular model, decreased p65 phosphorylation unequivocally corresponds to the loss of IKKα induction by TNFα in PGC-1 overexpressing cells and, interestingly, to diminished Akt activation. Akt-dependent phophorylation of p65 by IKK is an important mechanism to regulate p65 transactivation potential (36,46) and thus a good candidate to mediate the reduction observed (Fig. 8C). In addition to modulation of kinase activity, the involvement of a phosphatase is suggested by sustained phosphorylation of p65 after TNFα treatment for 5min even in the presence of PGC-1α and PGC-1β. As okadaic acid treatment did not abrogate differences between conditions, PP2A and PP1 are however unlikely to account for this effect. Therefore, further experiments are needed to determine the contribution of different phosphatases in this setting.

The transactivation potential of p65 is controlled by its phosphorylation status as it defines the affinity for cofactors important in suppressing or stimulating transcription of target genes (38). Decreased phosphorylation thus favors interaction with corepressors such as SMRT that decreases p65/p50 transactivation (47). Interestingly, SMRT levels are slightly induced by PGC-1α and

accordingly could contribute to lower cytokine expression. Stabilization of corepressor complexes on DNA-bound p65 is also fostered by nuclear receptor-mediated transrepression (39). For example, PPARα is able to exert anti-inflammatory action by ligand-dependent and –independent transrepressive mechanisms (48,49). We found a marked induction of PPARα by PGC-1α and PGC-1β, which presumably also contributes to negative regulation of pro-inflammatory cytokines by transrepression (Fig. 8C). This claim is substantiated by the reversal of the repressive PGC-1β effects on pro-inflammatory cytokine expression when PPARα was inhibited. PPARα regulates lipid metabolism and FFAs are able to serve as ligands for PPARα (50). Thus, the very pronounced suppressive effect of the PGC-1s on inflammatory gene expression observed after FFA treatment might reflect an additional ligand-dependent activation of PPARα leading to an even stronger transrepression in that experimental context.

Besides the reduction in p65 phosphorylation that is exerted by both PGC-1α and PGC-1β, only the latter was further found to repress p65 and p50 transcription in the basal state and accordingly the ability of these proteins to bind to DNA response elements. This offers an attractive explanation for the very low cytokine levels observed in the presence of PGC-1β. The NF-κB family members RelB and c-Rel were also suppressed transcriptionally in the basal state which puts PGC-1β in the position of a broader anti-inflammatory factor in skeletal muscle. Such anti-inflammatory potential was previously described only in macrophages, where PGC-1β is essential in alternative activation and ROS production (27,28). In contrast, PGC-1α does not alter expression of the classically activated / canonical NF-κB isoforms p65 and p50. However, PGC-1α overexpression induces transcript and protein levels of the alternative isoforms p100/p52 and RelB. This indicates a switch towards noncanonical / alternative NF-κB signaling. A recent publication outlined that alternative signaling via IKKα and RelB induces an oxidative phenotype in muscle driven by PGC-1β (51). Furthermore, activation of c-Rel and p50 was suggested to play a role in disuse atrophy (52). Finally, canonical p65 activation was linked to mitochondrial biogenesis in mouse embryonic fibroblasts and liver cells (53,54). All of these findings together with our data suggest a reciprocal, functional link between NF-κB signaling and oxidative metabolism. A potential induction of the alternative NF-κB pathway by PGC-1α in muscle therefore warrants further investigations.

Strikingly, inflammatory gene transcription was often selectively regulated by the two PGC-1 coactivators depending on the stimulus and the specific gene. These findings argue against a general repressive effect of PGC-1α and PGC-1β on tissue inflammation in muscle but rather indicate a specific, fine-tuned effect of these coactivators on NF-κB target genes. For example, while TLR1/2-induced cytokine production was suppressed by both PGC-1α and PGC-1β, only PGC-1α was able to block this production after treatment of the cells with a TLR4-specific ligand. Moreover, TLR6/2-induced TNFα expression was also diminished by PGC-1α, which was however not the case for TLR6/2-induced expression of IL-6. This probably reflects the activation of pathways other than NF-

κB downstream of TLRs that are not subject to regulation by PGC-1 or targeted by only one of the PGC-1 isoforms, respectively. Interestingly, while neither PGC-1α nor PGC-1β affected expression of TNF receptor 1 (TNFR1) (Suppl. Fig. 6A and 6B) both coactivators lowered mRNA levels of TLR1, TLR4 and TLR6 (Suppl. Fig. 6C and 6D). This downregulation might presumably contribute to the repressive effects observed with some TLR agonists. The expression pattern of TLR1, TLR4 and TLR6 (Suppl. Fig. 6C and 6D) further resembles genes in cluster 2 of the microarray, confirming a broader influence of PGC-1s on inflammatory genes. Moreover, the selectivity of the PGC-1s most likely reflects the distinct expression pattern as well as the distinct functions of PGC-1α and PGC-1β in the regulation of skeletal muscle physiology that will have to be further dissected in future experiments. In any case however, the concept that PGC-1s selectively and specifically repress inflammatory processes and thereby avoid the harmful consequences of broad and general cytokine suppression is compelling. Once understood in greater detail, the therapeutic potential of targeting PGC-1s to modulate specific inflammatory responses in muscle would be immense. Obviously, patients suffering from obesity and type 2 diabetes would profit from lower systemic inflammation mediated by rectified gene expression of PGC-1α and PGC-1β in skeletal muscle. Intriguingly however, muscle disorders like cachexia, muscular dystrophies, disuse atrophy or inflammatory myopathy also involve an inflammatory component with activation of the NF-κB pathway (55-58). In fact, chronic stimulation of the classical NF-κB pathway in muscle is sufficient to induce muscle wasting (5). Accordingly, mice heterozygous for p65 or harboring a genetic ablation of the IKKβ gene in the *mdx* background, a model for Duchenne muscular dystrophy, have improved pathology (59). Strikingly, ectopic elevation of PGC-1α in animal models for some of these diseases resulted in an amelioration of fiber damage and muscle functionality, e.g. in Duchenne muscular dystrophy, sarcopenia, a mitochondrial myopathy and denervation-induced fiber atrophy (20,21,44,60). Our data now suggest that at least part of the therapeutic effect of PGC-1α in these disease paradigms might stem from the anti-inflammatory effect. It is, thus, tempting to speculate that elevating PGC-1α and / or PGC-1β in muscle would also be beneficial in other conditions of muscle wasting by limiting the detrimental inflammatory component of the disease. In fact, muscle adaptation to endurance training that correlate with increased PGC-1α expression includes an increased resistance against fiber damage, tissue inflammation and, as a consequence, decreased exercise-induced muscle soreness. In contrast to PGC-1α, the implications of the repressive effect of PGC-1β on inflammatory gene expression remain less obvious until the physiological context of PGC-1β regulation and the function of this coactivator in muscle tissue have been more clearly delineated. Nevertheless, by virtue of their ability to reduce NF-κB activation, the PGC-1 coactivators are promising targets to antagonize inflammatory reactions in skeletal muscle associated with a large number of diseases.

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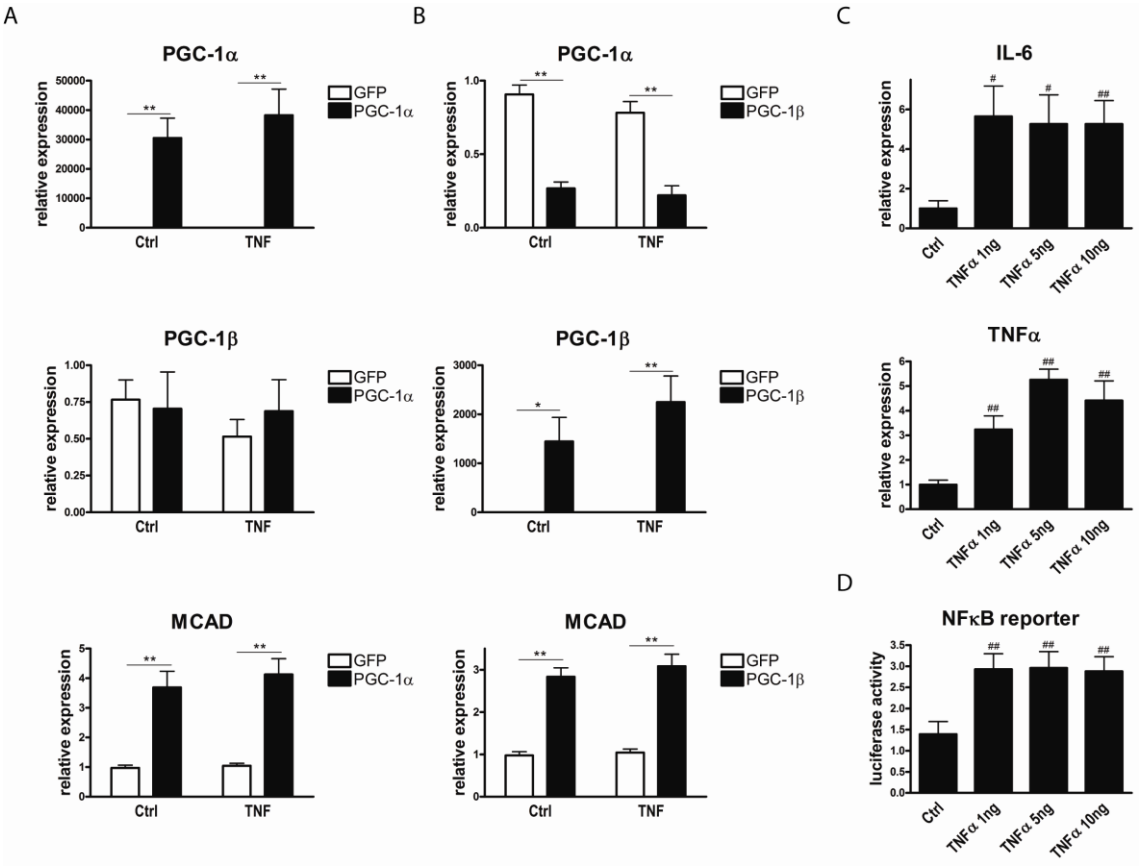
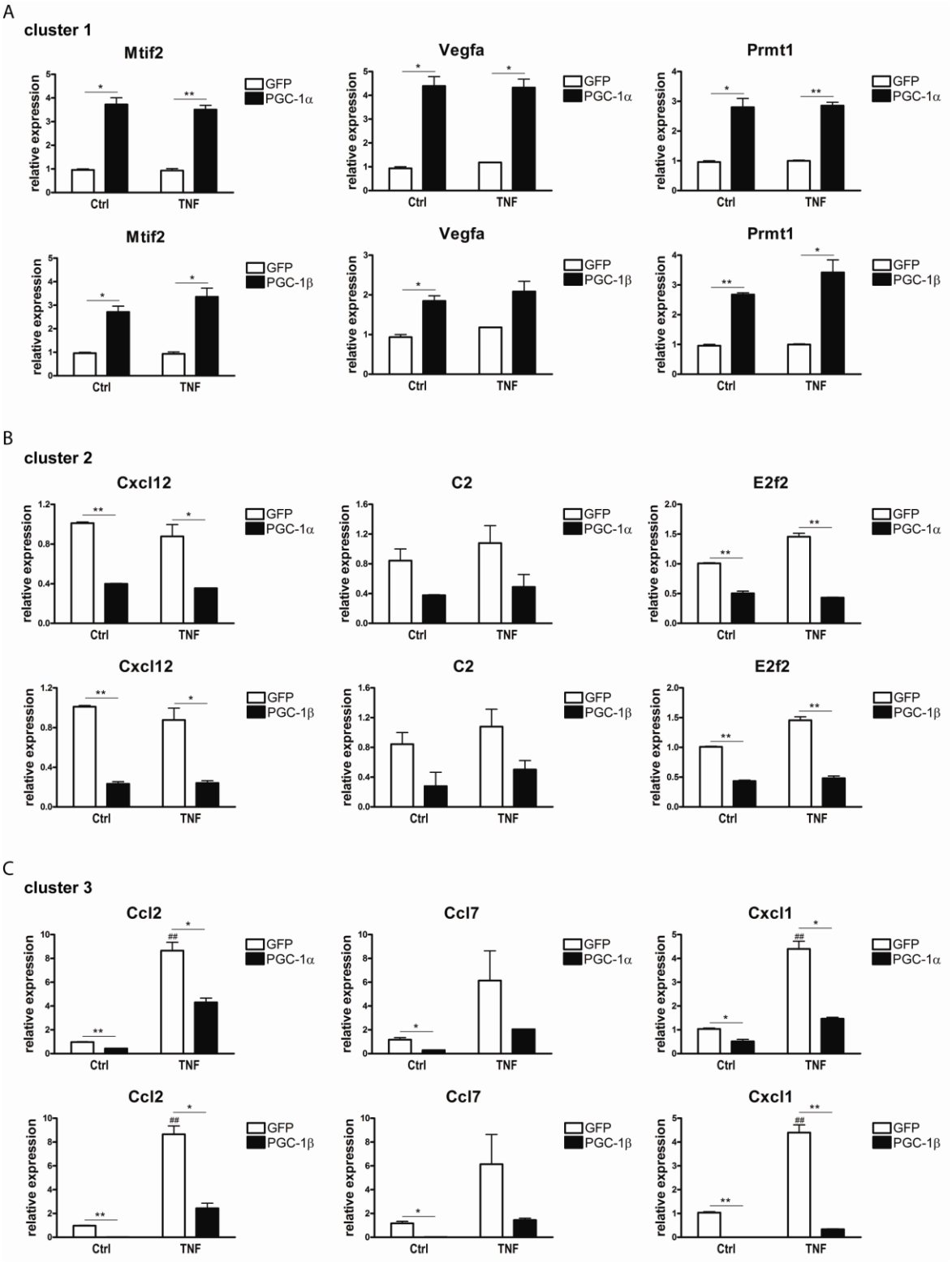
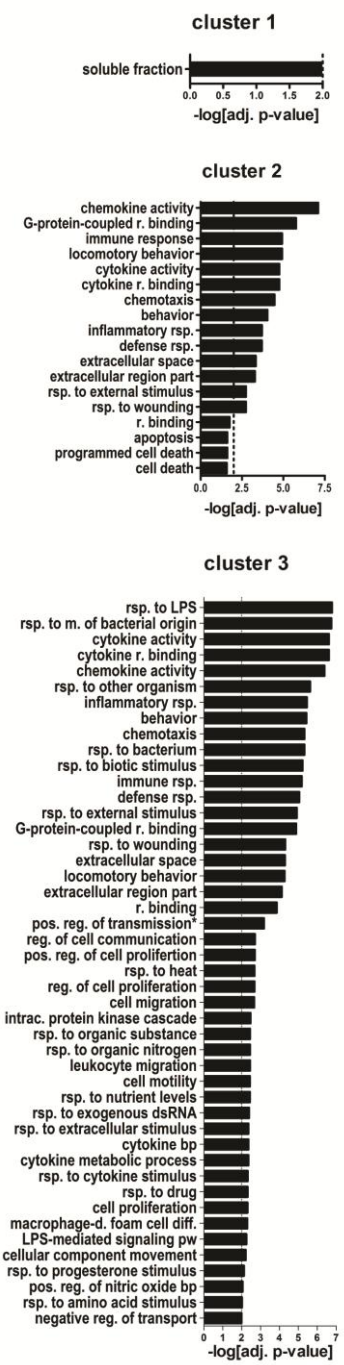
## 2.7 Supplemetal Material



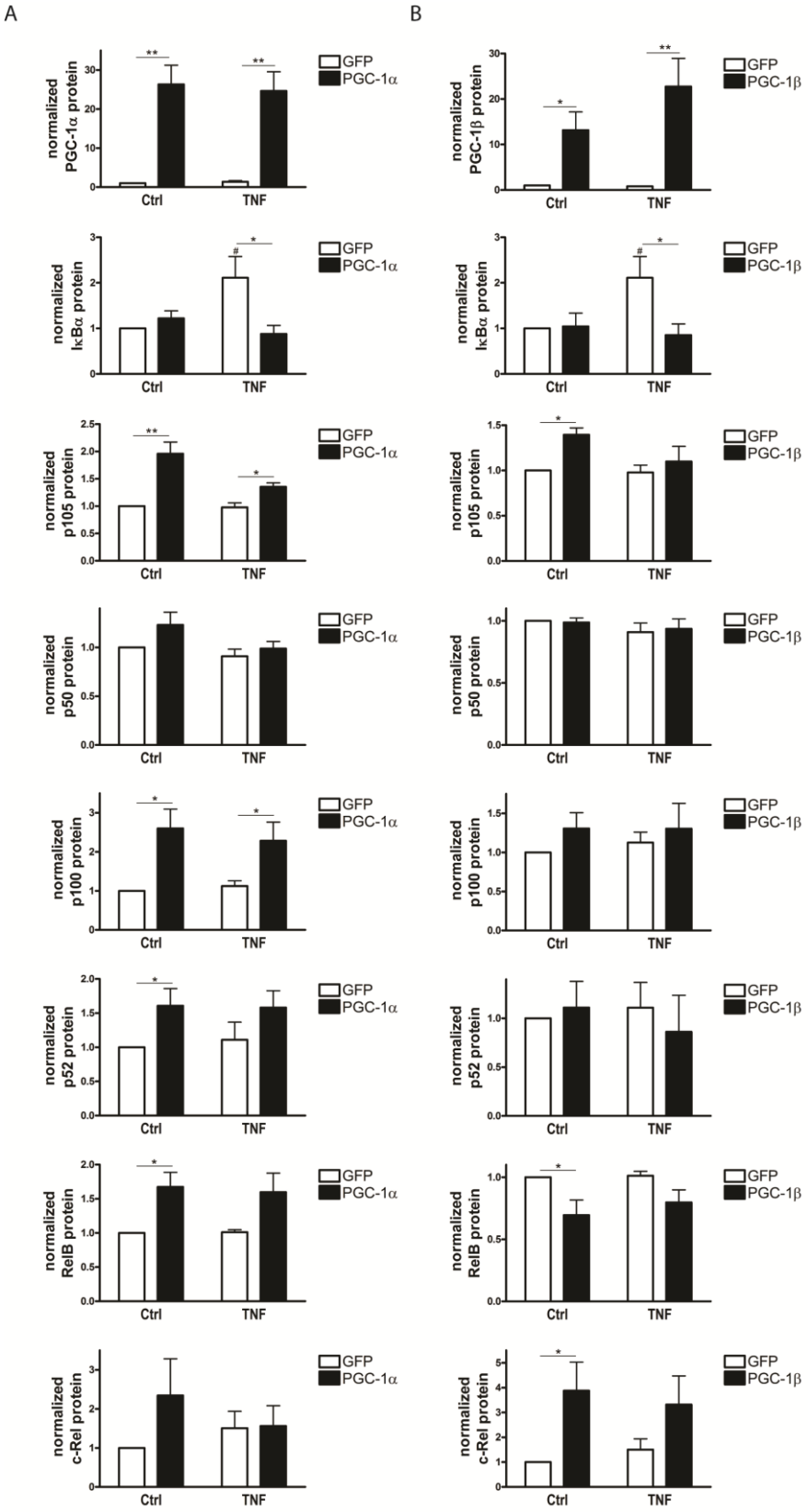

**Suppl. Figure 1** ***TNFα induces transcription of an NF−κB-controlled reporter gene similar to endogenous pro-inflammatory cytokines.*** **A, B,** Differentiated C2C12 myotubes overexpressing PGC-1α and GFP (Panel A) or PGC-1β and GFP (Panel B) were treated with TNFα for 2h. Expression of PGC-1α, PGC-1β and the PGC-1 target MCAD was determined by real-time PCR. Values represent the mean of at least 3 independent experiments * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1α/β versus GFP. **C**, C2C12 cells were differentiated and treated with different concentrations of TNFα for 2h. Expression of IL-6 and TNFα was assessed by real-time PCR. **D**, C2C12 cells were transfected with a NF−κB reporter construct (or a mutated reporter construct as control) and treated with different concentrations of TNFα for 2h. Luciferase activity was determined and is expressed as ratio of wt to mutated reporter gene expression. Values represent the mean of at least 3 independent experiments +SEM. # $P \leq 0.05$, ## $P \leq 0.01$ TNF versus Ctrl.




**Suppl. Figure 2** ***Confirmation of microarray results by real-time PCR.*** **A**, **B**, **C,** Differentiated C2C12 myotubes overexpressing PGC-1α or PGC-1β or GFP were treated with TNFα for 2h. Expression of three representative genes from each cluster of the microarray (Panel A - cluster 1, Panel B – cluster 2, Panel C – cluster 3) was determined by real-time PCR. # $P \leq 0.05$, ## $P \leq 0.01$ GFP TNF versus GFP Ctrl, * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1α/β versus GFP.

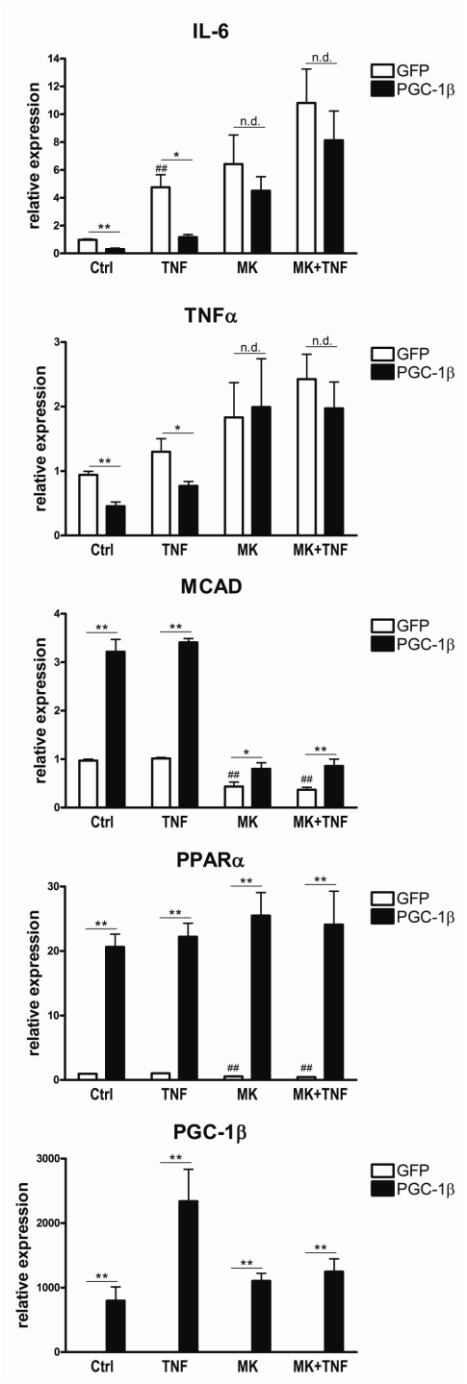



**Suppl. Figure 3** ***Inflammatory pathways are associated with PGC-1-repressed gene expression clusters.*** GO terms assigned to each cluster ($P<0.05$) are shown, dotted line indicates an adjusted p-value of 0.01. Abbreviations: bp=biosynthetic process, d.=derived, diff.=differentiation, intrac.=intracellular, m.=molecule, pos.=positive, pw=pathway, r.=receptor , reg.=regulation, rsp.=response, *of nerve impuls.

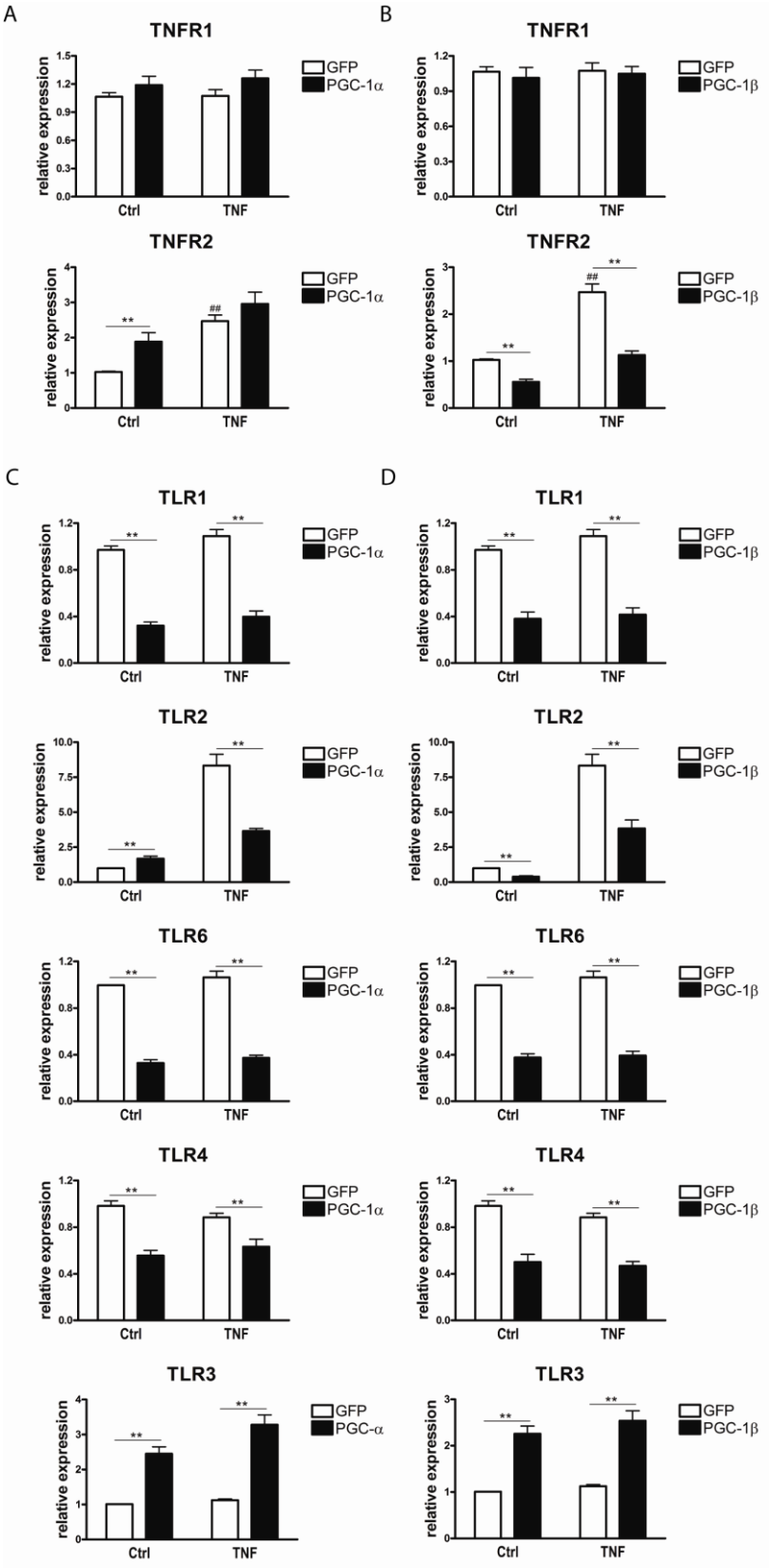



**Suppl. Figure 4** ***Changes in protein levels of NF−κB family members only partially explain repressive properties of the PGC-1s.*** **A**, **B**, Differentiated C2C12 myotubes overexpressing PGC-1α and GFP (Panel A) or PGC-1β and GFP (Panel B) were treated with TNFα for 2h. Quantification of protein levels of PGC-1 coactivators, IκBα and NF−κB family members, normalized to α-Tubulin (see Figure 6). Values represent the

mean of at least 3 independent experiments +SEM. # $P \leq 0.05$, ## $P \leq 0.01$ GFP TNF versus GFP Ctrl, * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1α/β versus GFP.




**Suppl. Figure 5** ***Inhibition of PPARα rescues PGC-1β-depressed pro-inflammatory gene expression.*** Differentiated C2C12 myotubes overexpressing PGC-1β and GFP were treated with TNFα for 2h in the presence or absence of the PPARα inhibitor MK 886. Expression of IL-6, TNFα, MCAD, PPARα and PGC-1β was determined by real-time PCR. Values represent the mean of at least 3 independent experiments +SEM. # $P \leq 0.05$, ## $P \leq 0.01$ GFP TNF versus GFP Ctrl, * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1α/β versus GFP.




**Suppl. Figure 6** ***PGC-1 coactivators reduce expression of relevant TLRs but not TNFR.*** **A, B, C, D,** Differentiated C2C12 myotubes overexpressing PGC-1α and GFP (Panel A and C) or PGC-1β and GFP (Panel B and D) were treated with TNFα for 2h. Expression of TNFR1 and TNFR2 (Panel A and B) and TLR1, TLR2, TLR3, TLR4 and TLR6 (Panel C and D) was determined by real-time PCR. * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1α/β versus GFP.

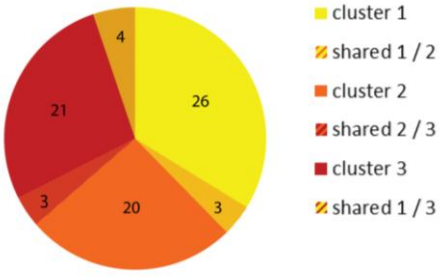
| Gene | Forward primer | Reverse primer |
|---|---|---|
| **C2** | GGTGACTGCTTTCTTGGGAG | GAGGTTCTCATCCGCGTTTA |
| **CBP** | TCAGACAAGACTGTGGAGGTCAAG | TTCCGACATCTCTCCAGAATCC |
| **Ccl2** | TTCCTCTTGGGGATCTTTTG | TCTGTGCCTGCTGCTCATAG |
| **Ccl7** | TCTCCAGCCTACTCATTGGG | AGGTCCCTGTCATGCTTCTG |
| **c-Rel** | TCGCAGTCTTCAATGTCCAG | TCAAAATTTGGGTATTCGGTG |
| **Cxcl1** | TCTCCGTTACTTGGGGACAC | ACCCAAACCGAAGTCATAGC |
| **Cxcl12** | TTTCAGATGCTTGACGTTGG | GCGCTCTGCATCAGTGAC |
| **E2f2** | GAAGAGGGTGTGACAGCTCC | GGGATCGCAGAGACCATAGA |
| **IL-6** | CCACGGCCTTCCCTACTTC | TTGGGAGTGGTATCCTCTGTGA |
| **MCAD** | AACACTTACTATGCCTCGATTGCA | CCATAGCCTCCGAAAATCTGAA |
| **MIP-1α** | TCCCAGCCAGGTGTCATTTT | TTGGAGTCAGCGCAGATCTG |
| **Mtif2** | AGTCTTCTTTGACTTGGGCTG | GACCTCGCCTGGGAGTTG |
| **NCoR1** | GACCCGAGGGAAGACTACCATT | ATCCTTGTCCGAGGCAATTTG |
| **p100/p52** | GAGCGTGATAAATGACGTGG | CACAGGACGAGAACGGAGAC |
| **p105/p50** | GAACGATAACCTTTGCAGGC | TTTCGATTCCGCTATGTGTG |
| **p65** | TAGGTCCTTTTGCGCTTCTC | GCTCCTGTTCGAGTCTCCAT |
| **PGC-1α** | TGATGTGAATGACTTGGATACAGACA | GCTCATTGTTGTACTGGTTGGATATG |
| **PGC-1β** | GGCAGGTTCAACCCCGA | CTTGCTAACATCACAGAGGATATCTTG |
| **PPARα** | GCGTACGGCAATGGCTTTAT | ACAGAACGGCTTCCTCAGGTT |
| **Prmt1** | GAAACTTCTTCAAGAGGCGG | GACTCGGGTGAAGATGGC |
| **RelB** | AGTCTTTCCCCACGAGGCTA | CCATCGAGCTTCGAGACTG |
| **SMRT** | CCTTCCGTGAGAAGTTTATGCA | CACACTCAGCGACCGTCTTTC |
| **TBP** | GGCCTCTCAGAAGCATCACTA | GCCAAGCCCTGAGCATAA |
| **TLR1** | GGCTTTGCAGGAACTCAATGTA | CCCCGCACCCAGGAA |
| **TLR2** | CCCTGTGCCACCATTTCC | CCACGCCCACATCATTCTC |
| **TLR3** | GATTCTTCTGGTGTCTTCCACAAA | AATGGCTGCAGTCAGCTACGT |
| **TLR4** | GCAGCAGGTGGAATTGTATCG | TGTGCCTCCCCAGAGGATT |
| **TLR5** | TCACCTGCCATTGATGTCCTT | ACGCAATAGGATGGAGGGAAA |
| **TLR5** | ACCTGGATGTCTCACACAATCG | GCCTCAGGCTCGCCATAG |
| **TLR7** | CAGTGAACTCTGGCCGTTGA | CAAGCCGGTTGTTGGAGAA |
| **TLR8** | GGAGACACTGCTACTGAGCCATAA | TCCTGGCTTCGGAGAGGAA |
| **TLR9** | AGCTGAACATGAACGGCATCT | CCAGCCATCTGAGCGTGTACT |
| **TNFα** | CACAAGATGCTGGGACAGTGA | TCCTTGATGGTGGTGCATGA |
| **TNFR1** | ATGGATGTATCCCCATCAGC | CTTCATTCACGAGCGTTGTC |
| **TNFR2** | AGATCTGGCACTCGTACCCA | GTCTTCGAACTGCAGCTGTG |
| **Vegfa** | CATCTTCAAGCCGTCCTGTGT | CTCCAGGGCTTCATCGTTACA |

**Suppl. Table 1** ***Real-time PCR primers.***

A

| Cluster 1 | | Cluster 2 | | Cluster 3 | |
|---|---|---|---|---|---|
| **motif** | **z score** | **motif** | **z score** | **motif** | **z score** |
| NFY{A,B,C} | 9.74 | IRF1,2,7 | 6.98 | NFKB1_REL_RELA | 6.67 |
| GFI1 | 4.9 | FOX{F1,F2,J1} | 4.46 | AR | 4.93 |
| PAX5 | 4.32 | PRDM1 | 4.23 | NFIX | 4.72 |
| RFX1 | 3.79 | NR5A1,2 | 4.22 | TBP | 4.06 |
| SOX17 | 3.73 | TBP | 4 | SPI1 | 3.84 |
| TP53 | 3.72 | MAFB | 3.47 | FOS_FOS{B,L1}_JUN{B,D} | 3.84 |
| CTCF | 3.48 | HNF4A_NR2F1,2 | 3.46 | CTCF | 3.32 |
| SOX2 | 3.4 | REST | 3.27 | AIRE | 3.01 |
| AIRE | 3.33 | FOXQ1 | 3.18 | T | 2.93 |
| HNF4A_NR2F1,2 | 3.32 | BACH2 | 3.14 | TLX1 | 2.82 |
| SP1 | 3.04 | RUNX1 | 2.9 | GFI1B | 2.69 |
| PITX1 | 3.04 | STAT1,3 | 2.74 | FOXP3 | 2.52 |
| PPARG | 3.03 | HOXA9_MEIS1 | 2.65 | STAT2,4,6 | 2.49 |
| MZF1 | 3.03 | E2F1 | 2.6 | EN1,2 | 2.47 |
| EVI1 | 2.89 | HMGA1,2 | 2.6 | VSX1,2 | 2.47 |
| SPZ1 | 2.72 | NKX2-1,4 | 2.59 | BACH2 | 2.42 |
| RXR{A,B,G} | 2.63 | PPARG | 2.5 | TGIF1 | 2.38 |
| ZNF423 | 2.63 | FOXA2 | 2.44 | CEBPA,B_DDIT3 | 2.31 |
| AR | 2.6 | DMAP1_NCOR{1,2}_SMARC | 2.25 | NFE2 | 2.3 |
| AHR_ARNT_ARNT2 | 2.58 | NFATC1 | 2.21 | PAX2 | 2.28 |
| ELK1,4_GABP{A,B1} | 2.5 | CDC5L | 2.15 | ZBTB16 | 2.24 |
| FOXD3 | 2.48 | ZNF423 | 2.13 | KLF4 | 2.22 |
| FOX{I1,J2} | 2.41 | NFIX | 2.12 | HIF1A | 2.19 |
| PBX1 | 2.35 | NR1H4 | 2.07 | HOX{A5,B5} | 2.17 |
| LMO2 | 2.29 | ESR1 | 2.05 | NR6A1 | 2.14 |
| BPTF | 2.26 | POU6F1 | 2.01 | TBX4,5 | 2.09 |
| MSX1,2 | 2.24 | | | ARNT_ARNT2_BHLHB2_MAX_MYC_USF1 | 2.04 |
| KLF4 | 2.18 | | | RXRA_VDR{dimer} | 2.01 |
| TLX2 | 2.16 | | | | |
| RORA | 2.08 | | | | |
| ZNF384 | 2.04 | | | | |
| GATA1 | 2.03 | | | | |
| PAX6 | 2 | | | | |

B




**Suppl. Table 2** ***Gene expression clusters are associated with a distinct set of transcription factor binding motifs.*** **A**, List of motifs overrepresented in the promoters of each cluster with a z score ≥2. **B**, Proportion of distinct and shared motifs between clusters. Numbers indicate absolute amount of motifs.

# 3 THE PGC-1 COACTIVATORS PROMOTE M2 POLARIZATION OF TISSUE MACROPHAGES IN SKELETAL MUSCLE (MANUSCRIPT 2)

Petra S. Eisele[1,2], Markus Beer[1] and Christoph Handschin[1,2]

[1]Biozentrum, Division of Pharmacology/Neurobiology, University of Basel, Basel, Switzerland,
[2]Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland.

**Keywords:** PGC-1; skeletal muscle; inflammation; macrophages

**Author contributions:** P.S.E. designed and performed all animal experiments (injections and treadmill running), cell culture experiments and subsequent analysis of blood, muscle specimen and cells (ELISA, histological stainings, cytokine expression), analyzed the data and wrote the manuscript, M.B. helped with injections of mice and treadmill running, especially muscle sampling and blood withdrawal, C.H. supervised the study and wrote the manuscript.

## 3.1 Abstract

An aging and increasingly obese population is confronted with a number of pathologies, many of which are accompanied by a persistent, sterile inflammation. While an altered immune cell content in adipose tissue is a major driver of inflammation in metabolic pathologies, less attention was given to immune reactions in muscle so far. Importantly, regular muscle use is both therapeutic and preventive against many chronic diseases. The transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) mediates most of the beneficial effects of exercise and its absence results in less endurant and inflamed muscles. Inversely, PGC-1α and PGC-1β are both downregulated in inactive skeletal muscle as well as in diseases that are associated with a sedentary life-style, including type 2 diabetes. We now tested if PGC-1α and PGC-1β have anti-inflammatory effects *in vivo* after exposure of muscle to lipopolysaccharide (LPS) and tumor necrosis factor α (TFNα) injection as well as eccentric exercise. Surprisingly, we observed a PGC-1-dependent change in macrophage polarization from M1 to M2 type with a concomitant alteration of the cytokine profile characterized by an increase in anti-inflammatory factors and a strong suppression of the pro-inflammatory interleukin 12 (IL-12). The anti-inflammatory environment in muscle that is promoted by the PGC-1s might contribute to the beneficial effects of these coactivators on muscle function and provides a molecular link underlying the tight mutual regulation of metabolism and inflammation.

## 3.2 Introduction

Many chronic diseases are fostered by a sedentary lifestyle and are associated with a subclinical, systemic inflammation, for example metabolic pathologies like obesity and type 2 diabetes, cardiovascular diseases such as atherosclerosis, but also neurodegenerative disorders including Alzheimer´s (1,2). The link between metabolic and immune dysregulation has been demonstrated in fat tissue with massive accumulation of macrophages in obese humans and rodents (3). Mechanistically, the expansion of fat cells under conditions of overnutrition alters the adipokine expression profile and the levels of free fatty acids, which subsequently activate adipose tissue macrophages (ATMs) (4-6).

Macrophages display high plasticity and can adopt different states on a continuous scale from highly inflammatory "M1" to anti-inflammatory "M2" cells. The broad spectrum of macrophages reflects their diverse roles in various processes and is regulated by different stimuli: M1 or "classically activated" macrophages release large quantities of pro-inflammatory cytokines, e.g. tumor necrosis factor α (TNFα), interleukin 6 (IL-6) and monocyte chemotactic protein 1 (MCP-1), and possess high microbicidal capacity according to their role in the innate immune response against pathogens (7,8). Exposure to T helper cell 1 (Th1) cytokines like interferon-γ or toll-like receptor (TLR) ligands primes macrophages for this pro-inflammatory M1 phenotype. In contrast, M2 or "alternatively activated" macrophages are polarized by Th2 cytokines including IL-4, IL-13, IL-10, or by immune complexes. These cells are implicated in tissue remodeling and repair, immunomodulation, the resolution of inflammation as well as allergy, parasite infection and the progression of some tumors (9). Therefore, they express a distinct set of chemokines, e.g. C-C motif chemokine 1 (CCL1), CCL22 or IL-1Ra, cell surface receptors e.g. CD206 / macrophage mannose receptor 1 (Mrc-1), CD163 or macrophage scavenger receptor 1 (MSR-1) / CD204, and have higher phagocytic activity (7).

In lean animals, ATMs are primarily of the M2 phenotype. Obesity changes adipokine secretion and subsequently skews macrophages towards an M1 phenotype. This initiates a vicious cycle of cytokine secretion, further macrophage recruitment into adipose tissue and an inflammatory reaction in the adipocytes themselves. Finally, insulin resistance, exacerbated lipolysis and systemic inflammation ensue (10-12). In skeletal muscle, the main site of glucose disposal in the body, the situation is less clear. Obesity gives rise to intramuscular adipose depots and, analogous to fat tissue, M1-type macrophage accumulation (3). Their contribution to the development of diet-induced insulin resistance was shown in mouse models with macrophage-specific deletion of IKKβ (12) or JNK (13), respectively, which renders two main inflammatory pathways in macrophages innocuous. Similarly, depletion of CD11c positive cells, which correspond to the main M1 population in muscle (14), reduces insulin resistance. In contrast, macrophage-specific expression of PPARγ, which is linked to suppression of inflammation and thus an M2 phenotype, is required to maintain normal insulin sensitivity in skeletal muscle (15). The balance of macrophage polarization in muscle and signals that

lead to dysregulation and finally inflammation and insulin resistance in this tissue have however not been explored in depth so far.

Insulin resistance and type 2 diabetes can be counteracted by regular exercise (16), which also limits systemic inflammation (17). An important factor that translates training-induced changes into metabolic adaptations in skeletal muscle is the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). By coactivating an array of nuclear receptors and transcription factors, PGC-1α induces genes involved in mitochondrial biogenesis and oxidative phosphorylation to enable a higher endurance capacity (18,19). Mice carrying an extra allele of PGC-1α in skeletal muscle (MCKα) consequently score better in running tests and exhibit a larger proportion of slow-twitch fibers than wild-type (WT) littermates (20). Transgenic overexpression of the related PGC-1 family member PGC-1β (MCKβ) also mediates lower fatigability and a switch towards IIX fibers in skeletal muscle (21). Inversely, skeletal muscle-specific deletion of PGC-1α results in poorer performance (22). This effect is aggravated by concomitant ablation of PGC-1β (23). Hence, both PGC-1s are drivers of oxidative metabolism with shared as well as distinct functions in skeletal muscle.

In human diabetic patients, the expression of PGC-1α and PGC-1β is reduced in skeletal muscle with coordinated depression of mitochondrial oxidative phosphorylation (24,25). Importantly, in these patients, PGC-1α levels inversely correlate with IL-6 or TNFα independent of body mass index (BMI) (26), which suggests anti-inflammatory action of the PGC-1s. Further evidence for such properties derives from the skeletal muscle-specific PGC-1α deletion model that exhibits elevated levels of tissue and systemic inflammation (26). In the present study, we therefore explored the role of the PGC-1s in skeletal muscle *in vivo* on local and systemic inflammatory events triggered by injection of inflammatory agents (LPS or TNFα) into tibialis anterior (TA) or a bout of muscle damage-inducing downhill running.

## 3.3 Material and Methods

### 3.3.1 Mice and treatments

C57BL/6 mice expressing PGC-1α ((27), MCKα) and FVB/N mice expressing PGC-1β ((21), MCKβ) under the control of the muscle creatine kinase (MCK) promoter were bred with respective WT mice to obtain WT and transgenic littermates. Male mice were maintained on a standard rodent chow with 12h light/dark cycle and subjected to experiments at 8 – 12 weeks of age. Injections were performed under sevoflurane anesthesia. Mice were randomly assigned to one experimental group and subsequently injected intramuscularly (*i. m.*) into TA with either PBS (30µl/TA), or LPS (2µg/TA) or TNFα (50ng/TA)in both legs. 4h post injection, mice were euthanized and blood as well as TAs collected for further analysis. Downhill running was performed on a motor-driven treadmill (Columbus Instruments) after 2 days of acclimatization at 5m/min with gradually declining slope. Mice were then randomly assigned to the exercise or sedentary control groups. The exercise group ran at a decline of -10° at alternating speeds (5min at 6m/min, 10min at 12m/min) for 2h (MCKα) or 3h (MCKβ) or until exhaustion. 24h post running, mice were euthanized and blood, TAs and quadriceps muscles collected for further analysis. All animal experiments were approved by the institutional and federal authorities.

### 3.3.2 Cell culture

The mouse myoblast cell line C2C12 was maintained below confluence in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum and 1x Penicillin/Streptomycin (Invitrogen). For differentiation into myotubes, growth medium was exchanged for DMEM supplemented with 2% horse serum (Invitrogen) for 3 days. PGC-1α, PGC-1β and GFP were overexpressed from recombinant adenoviral constructs 48h prior to treatment with TNFα (10ng/ml, Sigma-Aldrich) and LPS (40ng/ml, Sigma-Aldrich), which lasted for an additional 4h.

### 3.3.3 Semiquantitative real-time PCR

Muscle tissue was homogenized using matrix particles (Qbiogene) in a FastPrep FP120 cell disrupter (Thermo Scientific) and RNA isolated with Trizol (Invitrogen) while residual DNA

contamination was removed by DNase I (Invitrogen) digestion. 1µg of RNA was reverse transcribed with SuperScript II (Invitrogen) and the resulting cDNA used as template for RT-PCR. To detect relative expression levels cDNA was amplified with the SYBR Green Master mix (Applied Biosystems) and analyzed on a StepOnePlus RT-PCR System (Applied Biosystems). The respective primer pairs are listed in Suppl. Table 1. All values are normalized to the expression of TATA-Box binding protein (TBP) and expressed as fold induction over untreated WT animals.

### 3.3.4 ELISA

To determine serum cytokine concentrations, sandwich immunoassays for TNFα and IL-6 were performed according to the manufacturer's instructions (Quantikine, R&D Systems).

### 3.3.5 Histology

TA and quadriceps muscles were snap frozen in liquid nitrogen-chilled isopentane and cut into cross sections on a microtome/cryostat (Leica CM1950). Specimen were either stained with Mayer's hematoxylin and eosin (Sigma) and light microscopically analyzed or stained with immunofluorescence methods. For the latter, sections were fixed in paraformaldehyde (4%, 10min), washed (3 times with PBS), blocked (0.2% Triton X-100, 2% bovine serum albumin in PBS, 30min) and stained with specific antibodies against CD68 and CD206 (AbD serotec, 1:400, 1h). After washing, samples were immunofluorescently labeled with an Alexa Fluor 488-conjugated anti-rat IgG secondary antibody (Invitrogen, 1:100, 1h), washed again and mounted with DAPI-containing medium (Vectashield, Vector Laboratories). Macrophage staining was visualized on a fluorescent microscope (Leica DMI 4000 B) and images analyzed with the Imaris software (Bitplane).

### 3.3.6 Statistical analysis

Data were analyzed with Student's *t* test using $P<0.05$ as significance threshold.

## 3.4 Results

### 3.4.1 PGC-1α and PGC-1β do not alter systemic cytokine levels in inflammation

To delineate the role of skeletal muscle PGC-1 in inflammation, we exposed transgenic and WT animals to different inflammatory stimuli. Specifically, we injected LPS, TNFα or PBS as control *i.m.* into TA and analyzed blood and muscles 4h later. PGC-1α expression was not affected by the injections and remained about 6 fold above WT levels in muscles of MCKα mice (Fig. 1A). Similarly, PGC-1β levels were not changed by overexpressing PGC-1α in PBS-injected, but were reduced in LPS-treated mice (Fig. 1A). LPS injection also lowered PGC-1β expression in the MCKβ model, although PGC-1β levels remained considerably higher compared to WT controls (about 9 fold, Fig. 1B). PGC-1α levels in these animals were reduced to about a third of WT levels (Fig. 1B). Plasma

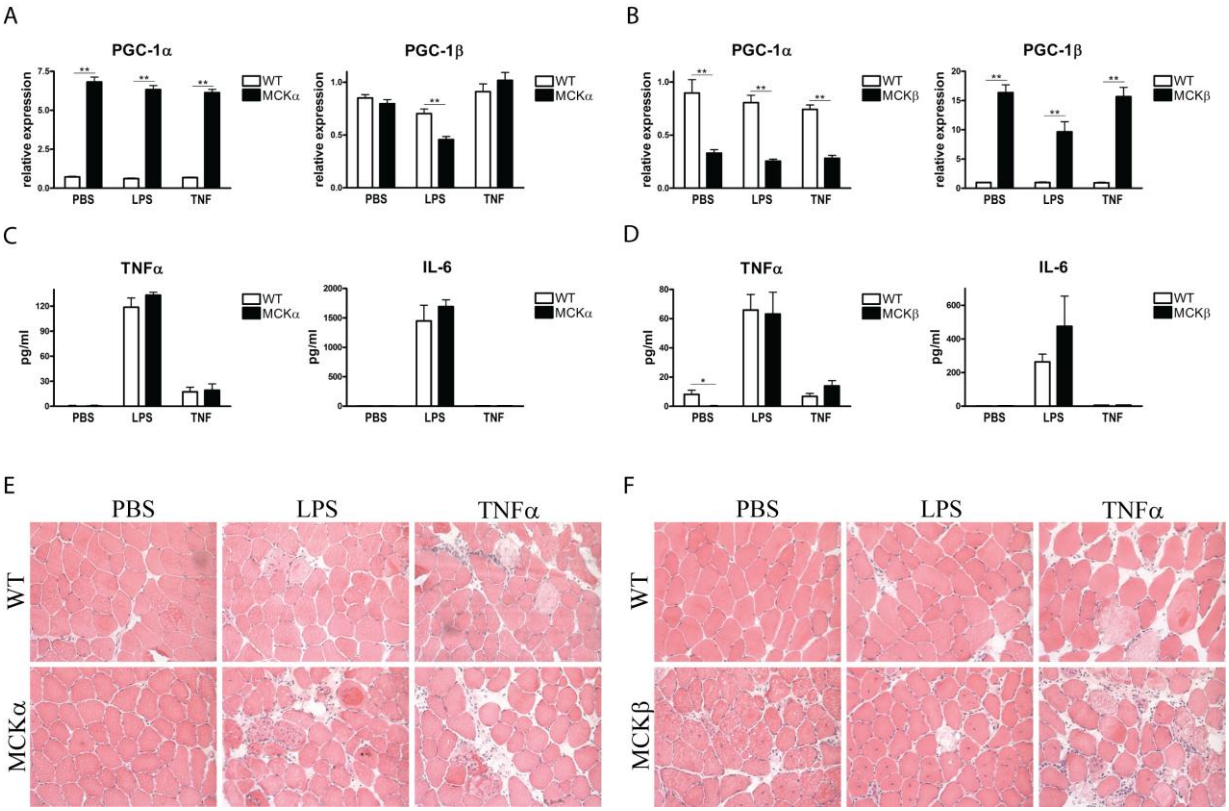



**Figure 1** ***PGC-1α and PGC-1β do not alter systemic cytokine levels after inflammatory stimulation.*** **A-F,** MCKα mice (Panel A, C, E) and MCKβ mice (Panel B, D, F) and respective WT controls were injected *i.m.* into TA with PBS, LPS or TNFα. **A, B,** mRNA expression levels of PGC-1 isoforms in TA were measured by RT-PCR. **C, D,** Serum levels of TNFα and IL-6 were determined 4h post injection. Values represent the mean of at least 7 animals +SEM. ** $P<0.01$, * $P<0.05$, WT versus MCK. **E, F,** TA cross sections were stained with hematoxylin and eosin 4h post injection for histological analysis. Representative images are shown.

levels of the pro-inflammatory cytokines TNFα and IL-6 were not detectable in PBS injected mice except for small amounts of TNFα in WT mice with FVB background from the breeding of the MCKβ mouse line (Fig. 1C, D). Transgenic expression of PGC-1β reduced plasma TNFα levels below detection limit. As expected, IL-6 and TNFα were found at high levels in the plasma of MCKα, MCKβ and the respective WT mice after LPS injection and at moderately elevated levels after TNFα injection. Skeletal muscle-specific overexpression of PGC-1α and PGC-1β, respectively, did not affect systemic TNFα or IL-6 levels after either stimulus (Fig. 1C, D).

### 3.4.2 PGC-1α and PGC-1β alter macrophage populations in skeletal muscle after injection of inflammatory agents

Since no evidence for an immunomodulatory role of the PGC-1s on systemic inflammation was found, we next focused our attention on tissue inflammation. Therefore, to assess immune cell infiltration of muscle tissue after injection of inflammatory stimuli, TA cross sections were first stained with H&E. MCKβ and to a smaller extend also MCKα mice harbored more non-muscle mononuclear cells already under basal conditions. LPS and TNFα both evoked a further substantial influx of immune cells compared to PBS-injected muscle (Fig. 1E, F). The difference between transgenic and WT animals persisted after injection of inflammatory agents, (especially in case of MCKβ) (Fig. 1E, F). The high number of regenerating fibers with centrally located nuclei in some areas of the TA in the MCKβ model finally indicated a basal, low level of continuous fiber damage and regeneration in these animals. This was not observed in MCKα mice.

To obtain a more detailed picture of the immune cell distribution in TA after injection of LPS and TNFα, mRNA expression of F4/80, which reflects the total amount of M1 and M2 macrophages in the muscle, was analyzed. In line with the H&E staining, F4/80 levels in TA of MCKβ mice were considerably higher than in WT animals (Fig. 2G). A similar, though qualitatively much lower increase was also present in MCKα mice after injection of LPS or TNFα (Fig. 2C). LPS and TNFα injection did not substantially alter macrophage infiltration in any of the experimental groups (Fig. 2C, G). As expected for the early time point of analysis post insult (4 h), the infiltrate after LPS and TNFα injection that is observed in H&E staining thus predominantly consists of neutrophils that rapidly invade injured muscle tissue. Importantly, potential changes in macrophage polarization are therefore likely to be attributed to activation of tissue-residing and not of newly infiltrating macrophages at the time of analysis.

To characterize polarization of tissue macrophages, TA cross sections were stained with specific antibodies against CD68 to capture M1 and against CD206 to reveal M2 macrophages. While

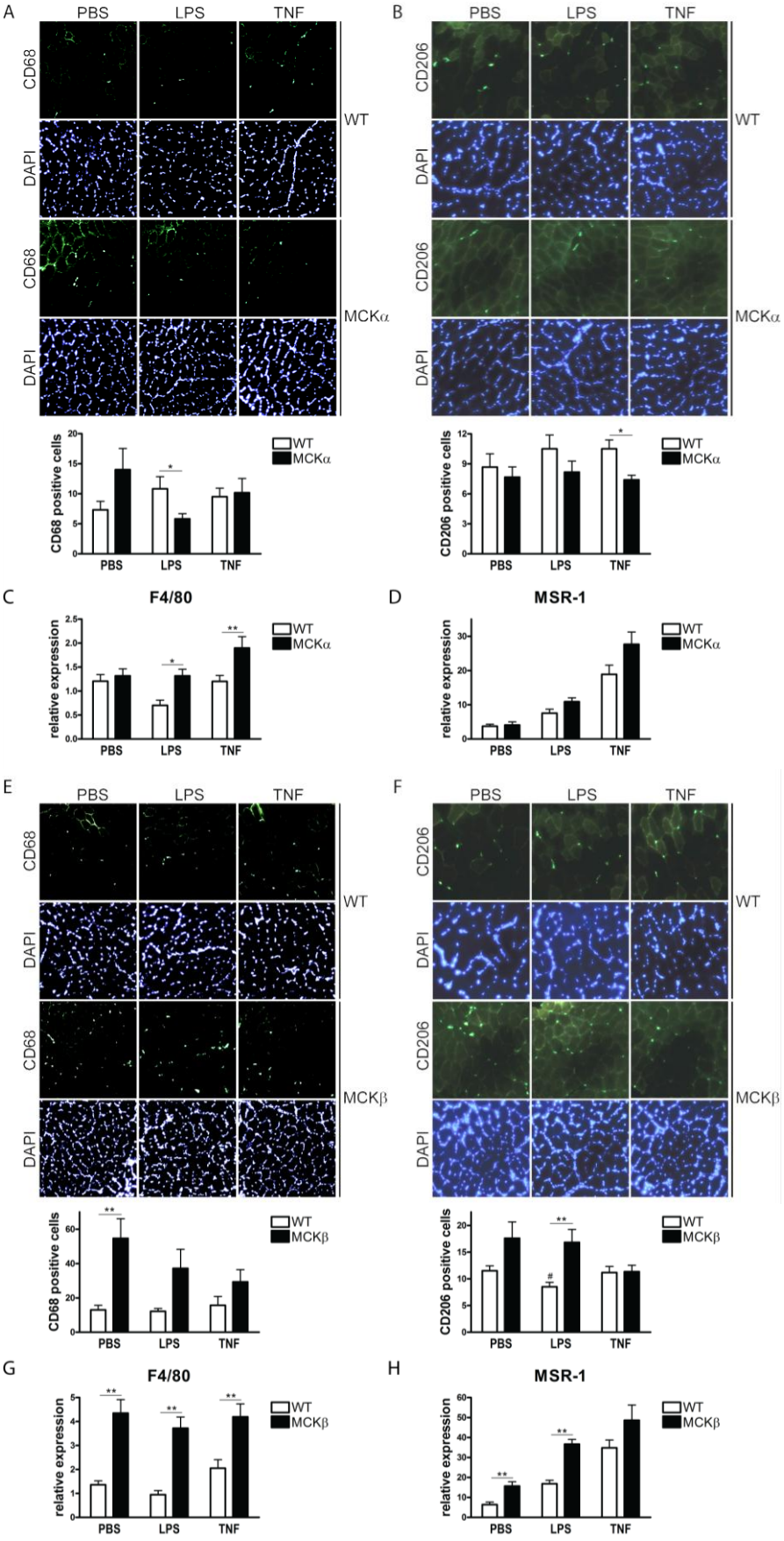



**Figure 2** ***PGC-1α and PGC-1β change macrophage populations in TA after injection of inflammatory agents.*** **A-H,** MCKα mice (Panel A-D) and MCKβ mice (Panel E-H) and respective WT controls were injected *i.m.* into TA with PBS, LPS or TNFα. **A, B, E, F,** Immunofluorescence stainings of TA cross sections with CD68 (Panel

A, E, M1), CD206 (Panel B, F, M2) and DAPI to detect macrophage populations 4h post injection. Representative images and quantifications are shown. **C, D, G, H,** Relative mRNA expression F4/80 (pan-macrophage marker) and MSR-1 (M2) in TA of injected MCKα (Panel C, D), MCKβ (Panel G, H) and WT mice was determined by RT-PCR. Values represent the mean of at least 7 animals +SEM. ** $P<0.01$, * $P<0.05$, WT versus MCK.

a non-significant trend for a higher CD68 signal was observed in PBS-injected MCKα mice, transgenic expression of PGC-1α reduced CD68-positive cell number in LPS-injected animals (Fig. 2A). Based on the CD206 staining, the number of M2 macrophages was not affected by muscle PGC-1α except for a lower M2 content after TNFα injection (Fig. 2B). Accordingly, expression of the M2 macrophage marker MSR-1 remained unchanged (Fig. 2D). Thus, in the experimental context of strong, acute inflammation, PGC-1α appears to influence mainly M1 activation, at least at the time of analysis.

F4/80 mRNA expression was consistently elevated by PGC-1β in muscle (Fig. 2G). Accordingly, MCKβ muscles contained significantly more M1 macrophages based on CD68 staining in PBS-injected mice. Interestingly, while a trend for higher M1 macrophages levels remained, the difference became non-significant in MCKβ mice after LPS- and TNFα injection, respectively (Fig. 2E). M2 macrophage polarization was significantly increased by transgenic PGC-1β after LPS injection whereas no difference was observed in TNFα-treated mice (Fig. 2F). Accordingly, higher expression of the M2 macrophage marker MSR-1 underlined the results obtained with CD206 staining in PBS- and LPS-treated animals (Fig. 2D, H).

### 3.4.3 PGC-1α and PGC-1β evoke an anti-inflammatory environment in skeletal muscle after injection of inflammatory agents

Based on the shift in macrophage polarization mediated by PGC-1α and PGC-1β after the injection of inflammatory agents, we next mapped the cytokine environment in TA in the different animal models. For that purpose, expression levels of pro-inflammatory cytokines associated with M1 activation and anti-inflammatory cytokines associated with M2 activation were determined. The M1 cytokines TNFα, IL-6, MIP-1α and MCP-1 were strongly induced in WT muscles by injection of inflammatory agents (Fig. 3A, C). However, skeletal muscle-specific overexpression of PGC-1α did not affect the elevation of these pro-inflammatory cytokines in stimulated muscle (Fig. 3A). Likewise, PGC-β did not change expression levels of these M1 cytokines (Fig. 3C). In stark contrast, the pro-inflammatory M1 cytokine IL-12 was potently suppressed by PGC-1α (Fig. 3A) and PGC-1β (Fig. 3C).

The M2 cytokines CCL1, CCL22, IL-1Ra and TGFβ were all significantly augmented by LPS and TNFα (Fig. 3B, D). IL-10 was only induced by LPS and not by TNFα at the concentration tested (Fig. 3B, D). PGC-1α did not alter the levels of CCL22, IL-1Ra and IL-10, but it clearly enhanced expression of CCL1 after LPS and TNFα injection compared to WT muscles (Fig. 3B). TGFβ expression was also elevated in MCKα mice across all experimental conditions (Fig. 3B). The M2 cytokines tested here were all upregulated by PGC-1β after PBS injection (Fig. 3D). This effect was potentiated after LPS injection except for TGFβ. TNFα did not elicit such clear differences between genotypes and only CCL1 expression was significantly higher in MCKβ muscles compared to WT (Fig. 3D). This cytokine expression pattern matches the smaller effects in macrophage polarization after TNFα injection as compared to LPS.

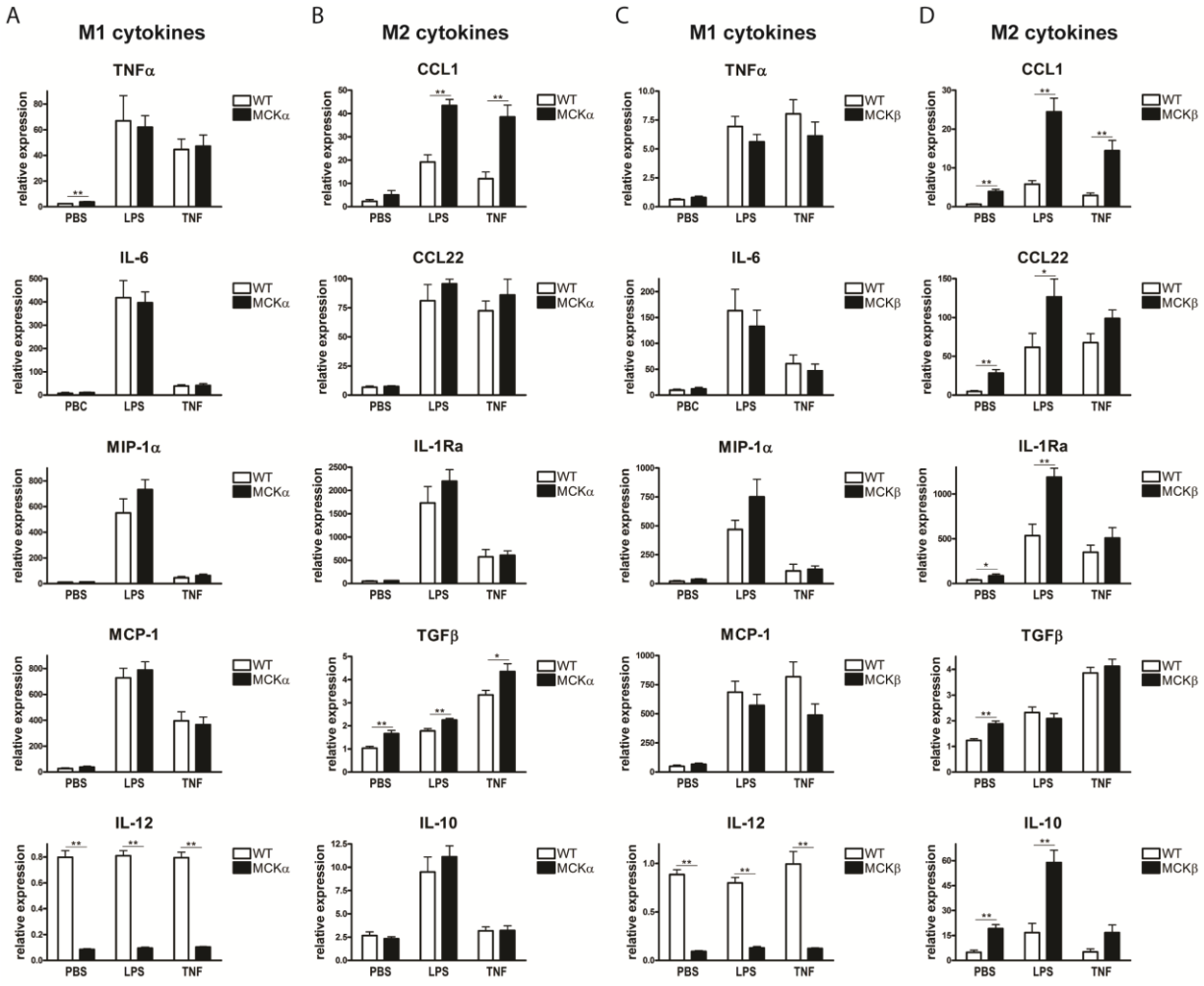



**Figure 3** ***PGC-1α and PGC-1β cause an anti-inflammatory environment in TA after injection of inflammatory agents.*** **A-D,** MCKα mice (Panel A, B) and MCKβ mice (Panel C, D) and respective WT controls were injected *i.m.* into TA with PBS, LPS or TNFα. Relative expression of different M1 (Panel A, C) and M2 cytokines (Panel B, D) in TA was analyzed by RT-PCR 4h post injection. Values represent the mean of at least 7 animals +SEM. ** $P<0.01$, * $P<0.05$, WT versus MCK.

### 3.4.4 PGC-1α and PGC-1β alter macrophage populations in skeletal muscle after downhill running

LPS- and TNFα-injection trigger a strong, acute immune reaction. However, based on their known function, the PGC-1s are not necessarily expected to exert general immunomodulatory effects when exclusively overexpressed in skeletal muscle. Therefore, to include an exercise paradigm that more closely resembles a pathophysiological process linked to the function of the PGC-1s in muscle, downhill running as a milder and more physiological inflammatory stimulus was examined next. As expected, MCKα animals were able to complete the downhill running protocol with a decline of -10° (see Materials and Methods) as their WT littermates (Suppl. Fig. 1A). Surprisingly, MCKβ mice performed significantly worse compared to their WT controls covering only about half the distance before reaching exhaustion (Suppl. Fig. 1B). Plasma IL-6 levels accordingly increased in the MCKβ model, but not in MCKα mice post-exercise (Suppl. Fig. 1C and 1D). Nevertheless, absolute levels were very low compared to LPS and TNFα injections confirming the more moderate inflammatory effect of downhill running. Systemic TNFα levels remained below detection limit in all sedentary and exercised groups.

Overexpression of PGC-1α in TA and quadriceps of the MCKα animals was about 8 fold and not changed by running in neither genotype (Suppl. Fig. 1E, F). PGC-1β overexpression in the MCKβ model was about 17 fold in both muscles (Suppl. Fig. 1G, H). PGC-1α expression was induced in WT muscles by exercise, reaching statistical significance only in quadriceps, but repressed in transgenic MCKβ muscles to about a third of WT levels (Suppl. Fig. 1G, H).

F4/80 transcript expression was higher in exercised MCKα animals compared to controls suggesting a higher number of total M1 and M2 macrophages (Fig. 4C). Importantly, the CD68-positive M1 macrophage cell number in MCKα mice was statistically not distinguishable from WT controls (Fig. 4A). In contrast, CD206-positive M2 macrophages remained higher in transgenic animals after running resulting in a marked difference between genotypes (Fig. 4B). MSR-1 expression analysis revealed a non-significant trend for higher levels in MCKα mice (Fig. 4D). Transgenic PGC-1α in TA thus fosters a stronger M2 polarization 24h after the inflammatory insult caused by eccentric muscle contractions.

In PGC-1β transgenic mice, total as well as M1 macrophage numbers were augmented compared to WT in sedentary and exercised animals as indicated by the higher F4/80 expression and CD68 staining, respectively (Fig. 4E, G). In these mice, M2 macrophage occurrence in TA was also increased (Fig. 4F). Accordingly, higher expression of the M2 macrophage marker MSR-1 was observed in MCKβ animals post exercise (Fig. 4H).

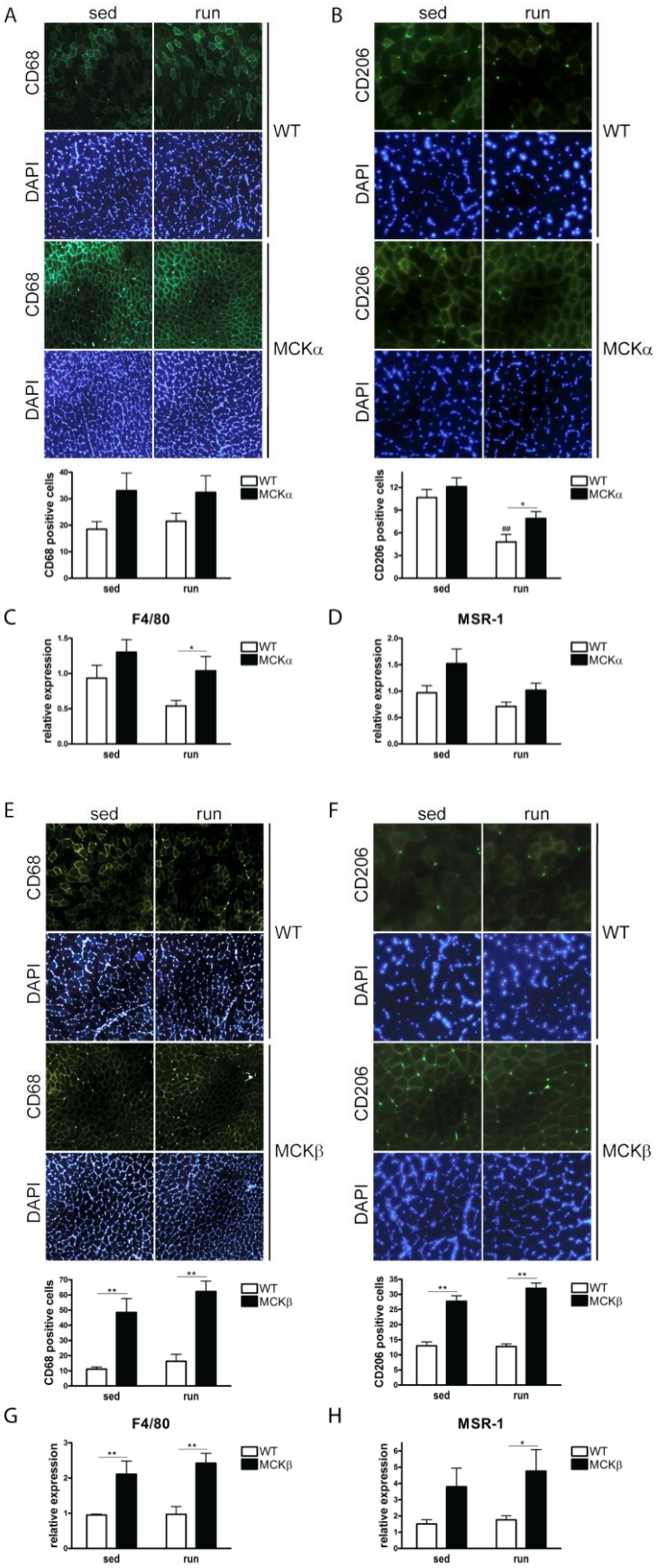



**Figure 4** ***PGC-1α and PGC-1β change macrophage populations in TA after downhill running.*** **A-H,** MCKα mice (Panel A-D) and MCKβ mice (Panel E-H) and respective WT controls were running on a treadmill at a decline of 10°. **A, B, E, F,** Immunofluorescence stainings of TA cross sections with CD68 (Panel A, E, M1),

CD206 (Panel B, F, M2) and DAPI to detect macrophage populations 24h post injection. Representative images and quantifications are shown. **C, D, G, H,** Relative mRNA expression of F4/80 (pan-macrophage marker) and MSR-1 (M2) in TA of sedentary and exercised MCKα (Panel C, D), MCKβ (Panel G, H) and WT mice was determined by RT-PCR. Values represent the mean of at least 6 animals +SEM. ** $P<0.01$, * $P<0.05$, WT versus MCK. Abbreviations: sed = sedentary, run = exercised.

### 3.4.5 PGC-1α and PGC-1β cause an anti-inflammatory environment in skeletal muscle after downhill running

To complement the quantification of macrophage polarization in the downhill running paradigm, we again characterized the cytokine environment in TA and quadriceps 24h post running. In the MCKα model, IL-6 expression remained unchanged in all experimental conditions (Fig. 5A). The pro-inflammatory cytokines MIP-1α and MCP-1 were elevated in sedentary MCKα muscle, while this difference vanished upon running. Interestingly, IL-12 and TNFα expression levels were repressed by PGC-1α in exercised mice, the former even in sedentary animals (Fig. 5A). Similar findings were obtained in the analysis of the quadriceps muscle (Suppl. Fig. 3A). In contrast to the mixed M1 macrophage gene expression pattern, M2 cytokines were augmented in sedentary PGC-1α transgenic TA and quadriceps (Fig. 5B and Suppl. Fig. 3B). Expression of CCL22 remained elevated in exercised TA and quadriceps of MCKα mice ( Fig. 5B and Suppl. Fig. 3B) while in TA CCL1 and TGFβ induction further persisted after down-hill running (Fig. 5B).

In MCKβ animals, MIP1α expression was increased compared to WT mice while TNFα levels were not significantly altered (Fig. 5C). In the exercised groups, MIP-1α and MCP-1 were elevated in MCKβ animals, diametrically opposite to the reduced levels of IL-6. Strikingly, IL-12 expression was strongly inhibited by PGC-1β in both groups. Again, analysis of the quadriceps muscle confirmed the mixed expression pattern of M1 macrophage-type genes controlled by PGC-1β (Suppl. Fig. 3C). More coherently, the M2 cytokines CCL1, CCL22, IL-1Ra and IL-10 were all elevated by PGC-1β in both muscles of sedentary animals (Fig. 5D and Suppl. Fig. 3D). In addition to these genes, TGFβ was also induced by PGC-1β post-exercise (Fig. 5D and Suppl. Fig. 3D). PGC-1α and PGC-1β in skeletal muscle therefore clearly favour the expression of a panel of anti-inflammatory cytokines corresponding to M2-type macrophage activation.

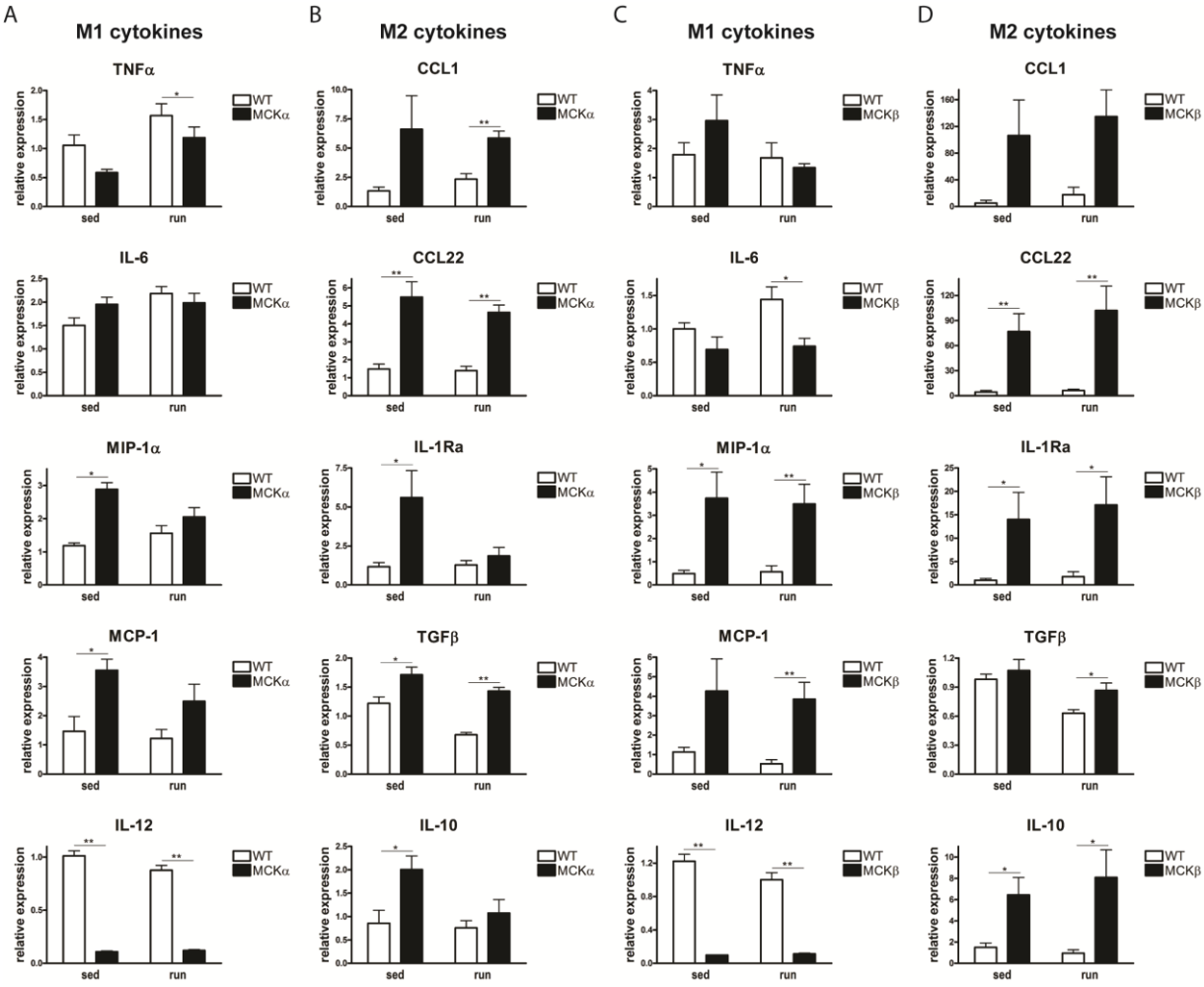



**Figure 5** ***PGC-1α and PGC-1β cause an anti-inflammatory environment in TA after downhill running.*** **A-D,** MCKα mice (Panel A, B) and MCKβ mice (Panel C, D) and respective WT controls were running on a treadmill at a decline of 10°. Relative expression of different M1 (Panel A, C) and M2 cytokines (Panel B, D) in TA was analyzed by RT-PCR 24h post running. Values represent the mean of at least 6 animals +SEM. ** $P<0.01$, * $P<0.05$, WT versus MCK. Abbreviations: sed = sedentary, run = exercised.

### 3.4.6 PGC-1α and PGC-1β suppress IL-12 in isolated skeletal muscle cells after treatment with inflammatory agents

Due to the high complexity of skeletal muscle tissue in vivo, it was impossible to distinguish muscle-derived cues from those produced and secreted by cells of the immune system in our experimental settings. To delineate the direct effects of the PGC-1s on muscle fibers distinctly from secondary effects on macrophages and other immune cells, we treated C2C12 myotubes overexpressing PGC-1α or PGC-1β with LPS or TNFα to mimic the injection experiment. We subsequently determined the mRNA expression of IL-12, IL-1Ra, TGFβ, CCL22, CCL1 and IL-10 by semiquantitative real-time PCR. The expression levels of CCL1 and IL-10 were below detection limit and thus most likely derived from immune cells *in vivo*. IL-1Ra, TGFβ and CCL22 were traceable;

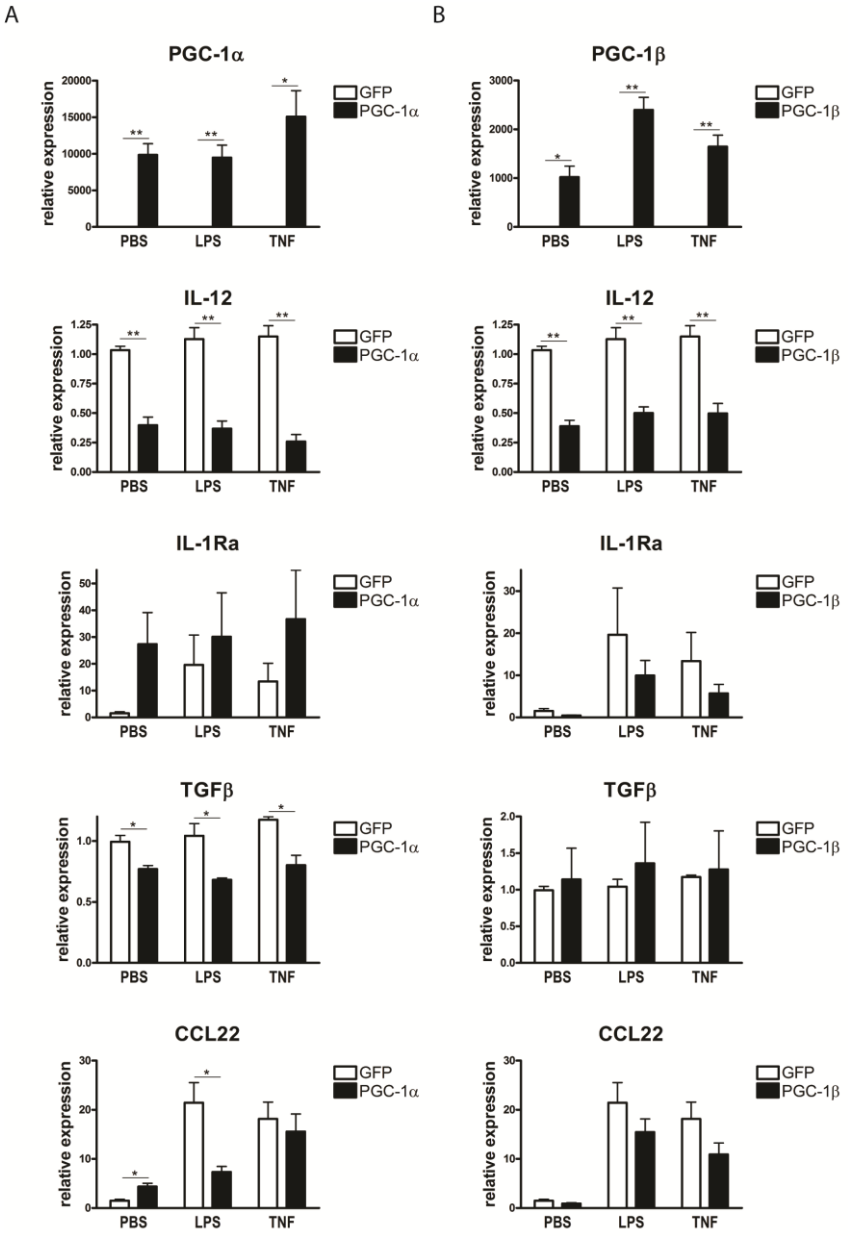



**Figure 6** ***PGC-1α and PGC-1β suppress IL-12 in skeletal muscle cells after treatment with inflammatory agents.*** **A, B,** C2C12 myotubes adenovirally overexpressing PGC-1α (Panel A), PGC-1β (Panel B) or GFP as control were treated with PBS, LPS or TNFα for 4h. Relative expression of different cytokines was determined by RT-PCR. Values represent the mean of at least 3 independent experiments +SEM. ** $P<0.01$, * $P<0.05$, WT versus PGC-1.

however, they were not regulated in the same way as *in vivo* indicating that neither PGC-1α nor PGC-1β boosted their expression after an inflammatory insult directly in the muscle cell (Fig. 6A, B). Importantly however, the strong suppression of IL-12 was recapitulated in the cellular system by overexpression of PGC-1α or PGC-1β (Fig. 6A, B). Thus, the very low IL-12 levels detected in muscle of transgenic mice are likely due to very low expression of this cytokine in muscle cells themselves. In contrast, the anti-inflammatory cytokines that are induced in transgenic muscle *in vivo* are presumably released from immune cells that reside in the muscle.

## 3.5 Discussion

Metabolism and inflammation are two biological processes that are tightly linked. We now report that the PGC-1 coactivators PGC-1α and PGC-1β constitute a molecular link between metabolism and inflammation in skeletal muscle. Strikingly, PGC-1 coactivators altered the balance of M1/M2 macrophages in skeletal muscle from M1 to M2 cells accompanied by a profoundly altered muscle cytokine profile. In particular, anti-inflammatory factors like CCL1, CCL22, IL-1Ra, TGFβ or IL-10 were induced in one or both MCK models depending on the experimental context, thereby creating an environment favoring M2 activation. Inversely, the pro-inflammatory M1 cytokine IL-12 was strongly suppressed in both models and this suppression could be replicated *in vitro* in C2C12 cells. It is therefore likely that the lack of IL-12 is a direct consequence of muscle-specific overexpression of either PGC-1. Studies in mice with an IL-12 deletion showed deficits in type 1 cytokines and more importantly, a bias in macrophage activation towards M2 (28,29), which supports our hypothesis that defective IL-12 production causes an altered macrophage polarization.

Systemically, we did not find a difference between PGC-1 overexpressing and WT animals at the time-points chosen as plasma levels of TNFα and IL-6 were comparable after the challenge. PGC-1s in skeletal muscle are therefore not able to suppress an acute inflammatory reaction of the immune system on whole-body level. A recent study with the MCKα animals that were injected *i.p.* with LPS yielded similar results: Plasma IL-6 and TNFα levels were not different between genotypes after LPS injection even though there was a reduction in basal TNFα levels (30). This is reminiscent of the situation in our MCKβ model. However, it remains to be determined if PGC-1α and PGC-1β in skeletal muscle are beneficial in conditions of chronic systemic inflammation, e.g. obesity-related morbidities, where cytokine levels are only slightly but permanently elevated as opposed to the acute, strong but transient inflammatory response in both the above mentioned and our study.

Classifying macrophages as M1 or M2 is a useful but simplified model, as there is a continuous spectrum in-between and several types of alternatively activated macrophages have been described. These are also referred to as M2a (alternative), M2b (type II) and M2c (deactivated) (31). M2a macrophages are induced by IL-4 and IL-13, produce IL-10, Il-1Ra and CCL22 among others and are involved in Th2 responses, allergy and parasite defense (32). M2b macrophages are induced by TLR or IL-1R ligands (e.g. LPS) together with immune complexes, are characterized by low IL-12 and high IL-10 levels and produce CCL1 which primes T cells towards Th2 and Tregs (33,34). Like M2b macrophages, M2c cells also serve immunoregulatory purposes. Induced by IL-10, they further amplify this signal and are the main players in tissue remodeling by their ability to produce TGFβ (31). We could not detect substantial amounts of IL-4 or IL-13 in muscle and it appears that the PGC-1s, in particular PGC-1α, favor M2b macrophage specification based on the low IL-12 and high CCL1 levels (34). It will be interesting to evaluate M2 numbers at a time-point later than 4h post injection to

follow up priming of these M2b cells in the injection model. The downhill running experiment provides evidence for this hypothesis as there are more M2 macrophages after running in MCKα muscles compared to WT 24h after the exercise. We however do not know if there are immune complexes involved in the activation as has been described for this macrophage type (33). How these M2b cells influences other cells of the innate and adaptive immune system notably via CCL1 in the course of the inflammation would also be of great interest as there were no T cells or eosinophils detectable at these early time-points. PGC-1α might also prime towards M2c as higher TGFβ levels were found throughout the experiment, yet IL-10 levels did not differ. PGC-1β seems to affect a broader spectrum of M2 macrophages as signature cytokines of all 3 types were detected.

Surprisingly, although the MCKβ mice have been reported to exhibit a higher endurance capacity than WT animals in an exercise protocol with a positive incline (21), these animals performed significantly worse during down-hill running in our hands. Obviously, PGC-1β might affect muscle function differently in concentric compared to eccentric contractions. Intriguingly however, signs of fiber damage and inflammation were detected even in sedentary MCKβ mice. It is thus conceivable that the supraphysiological expression of the PGC-1β transgene in these animals confers some detrimental effects on muscle fibers, similar to the pathological changes described in higher-expressing muscle-specific PGC-1α transgenic lines (27,35). In contrast, normal running behavior and much fewer signs of fiber damage and inflammation were detected in the MCKα mice. Importantly, despite these potentially confounding effects of increased M1-type macrophage activation and inflammation intrinsic to the animal models, PGC-1α and PGC-1β are still able to promote M2 type macrophage activation and an anti-inflammatory cytokine environment.

PGC-1α was shown to substantially improve the pathology of Duchenne muscular dystrophy and other muscle wasting diseases (36). Injured and dystrophy muscles also depend on M2 macrophages to undergo repair. After a first phase in which necrotic muscle fibers are removed by M1 cells, M2c macrophages govern the second phase of regeneration (37,38). Given the beneficial effect of physiological PGC-1α overexpression in Duchenne muscular dystrophy and its ability to shift the balance towards less M1 or more M2 cells after inflammatory insults as shown here, it is tempting to speculate that PGC-1α and maybe PGC-1β are instrumental in muscle regenerative processes by modulating immune responses that are vital to proper recovery.

In conclusion, the results presented here prove that PGC-1 coactivators are not only fundamental to control metabolism in muscle cells but also have a profound effect on the cytokine environment in this tissue. Due to crosstalk between muscle and immune cells, the balance of macrophages is skewed after an inflammatory insult from M1 to M2 cells providing a predominantly anti-inflammatory and immunomodulatory context. The consequences of this changed M1/M2 ratio in conjunction with obesity, diabetes and other diseases that are accompanied by a low-grade inflammation will be the subject of future studies. The possibility of influencing this ratio by

modulating PGC-1s bears tremendous potential to delay or even prevent the development of insulin resistance that requires pro-inflammatory M1 cells. Thus pharmacological targeting of the immunomodulatory effect of the PGC-1s in muscle and thereby promoting an M2 tissue macrophage phenotype could be a novel strategy for metabolic and muscle wasting diseases.

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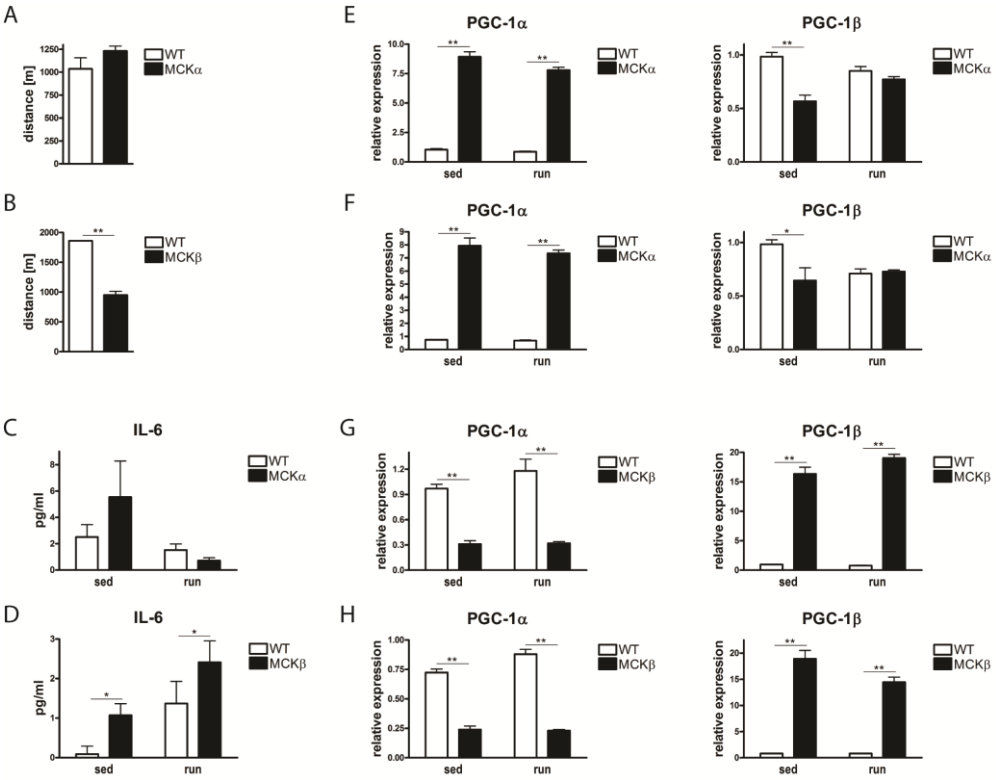
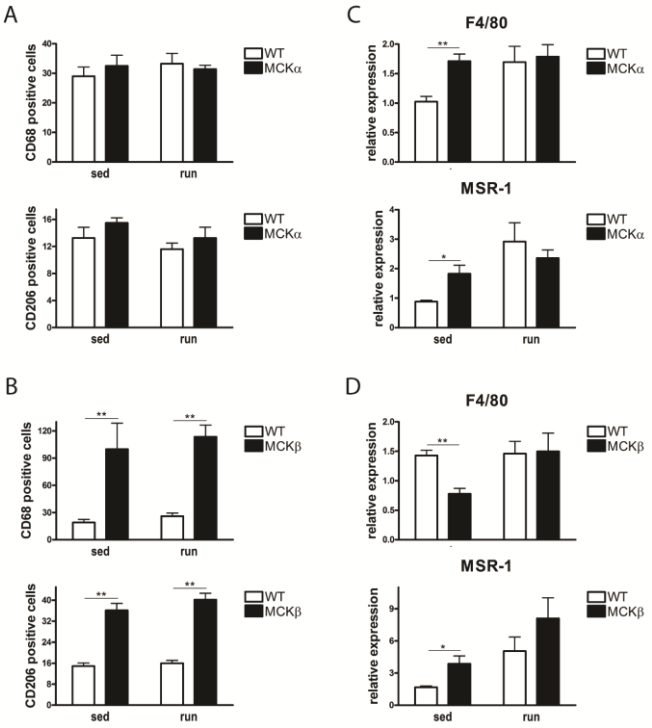
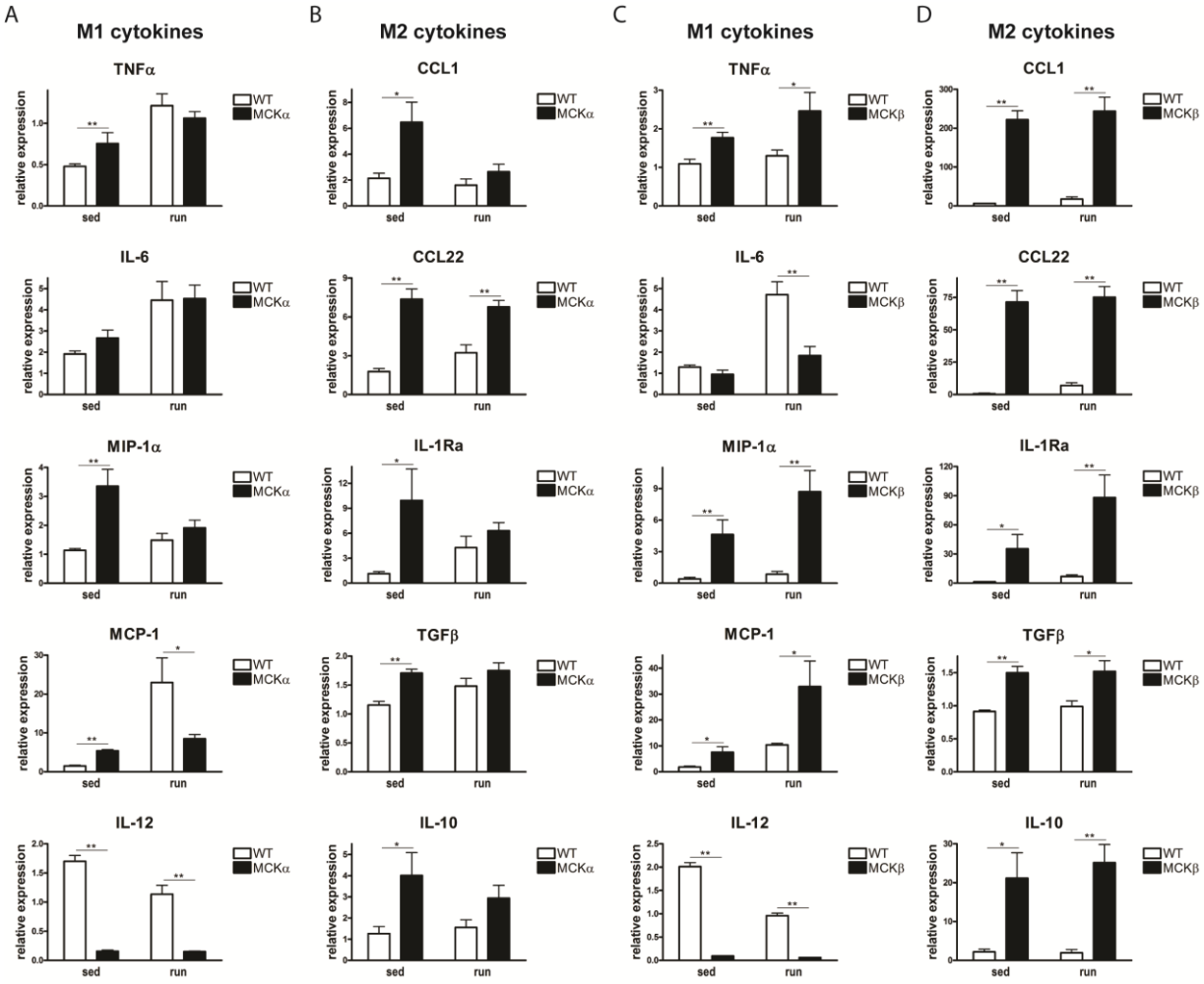
## 3.7 Supplemental Material




**Suppl. Figure 1** ***PGC-1α and PGC-1β do not suppress systemicIL-6 levels.*** **A-H,** MCKα mice (Panel A, C, E, F) and MCKβ mice (Panel B, D, G, H) and respective WT controls were running on a treadmill at a decline of 10°. **A, B,** Total distance covered by the different genotypes until reaching exhaustion or the end of the protocol is displayed. **C, D,** Serum levels of IL-6 were determined 24h post running. **E-G,** mRNA expression levels of PGC-1 isoforms in TA (Panel E, G) and quadriceps (Panel F, H) were measured by RT-PCR. Values represent the mean of at least 6 animals +SEM. ** $P<0.01$, * $P<0.05$, WT versus MCK. Abbreviations: sed = sedentary, run = exercised.




**Suppl. Figure 2** ***PGC-1β but not PGC-1α changes macrophage populations in quadriceps after downhill running.*** **A-D,** MCKα mice (Panel A, C) and MCKβ mice (Panel B, D) and respective WT controls were running on a treadmill at a decline of 10°. **A, B,** Quantification of immunofluorescence stainings of quadriceps cross sections with CD68 (M1) and CD206 (M2) to detect macrophage populations 24h post running. **C, D,** Relative mRNA expression of F4/80 (pan-macrophage marker) and MSR-1 (M2) in quadriceps of sedentary and exercised MCKα, MCKβ and WT mice was determined by RT-PCR. Values represent the mean of at least 6 animals +SEM. ** $P<0.01$, * $P<0.05$, WT versus MCK. Abbreviations: sed = sedentary, run = exercised.




**Suppl. Figure 3** ***PGC-1α and PGC-1β cause an anti-inflammatory environment in quadriceps after downhill running.*** **A-D,** MCKα mice (Panel A, B) and MCKβ mice (Panel C, D) and respective WT controls were running on a treadmill at a decline of 10°. Relative expression of different M1 (Panel A, C) and M2 cytokines (Panel B, D) in quadriceps was analyzed by RT-PCR 24h post running. Values represent the mean of at least 6 animals +SEM. ** $P<0.01$, * $P<0.05$, WT versus MCK. Abbreviations: sed = sedentary, run = exercised.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| **CCL1** | ACAGGAGGAGCCCATCTTTC | CTGCCGTGTGGATACAGGAT |
| **CCL22** | TGGAGTAGCTTCTTCACCCA | TCTGGACCTCAAAATCCTGC |
| **CD206** | GTGGATTGTCTTGTGGAGCA | TTGTGGTGAGCTGAAAGGTG |
| **CD68** | CCAATTCAGGGTGGAAGAAA | CTCGGGCTCTGATGTAGGTC |
| **CXCL-10** | CTTCCCTATGGCCCTCATTC | AAGTGCTGCCGTCATTTTCT |
| **F4/80+** | CTTTGGCTATGGGCTTCCAGTC | GCAAGGAGGACAGAGTTTATCGTG |
| **IL-1Ra** | AAATCTGCTGGGGACCCTAC | TGAGCTGGTTGTTTCTCAGG |
| **IL-10** | CTGGACAACATACTGCTAACCG | GGGCATCACTTCTACCAGGTAA |
| **IL-12** | GCTTCTCCCACAGGAGGTTT | CTAGACAAGGGCATGCTGGT |
| **IL-6** | CCACGGCCTTCCCTACTTC | TTGGGAGTGGTATCCTCTGTGA |
| **MCP-1** | CCCAATGAGTAGGCTGGAGA | TCTGGACCCATTCCTTCTTG |
| **Mgl1** | CAGGATCCAGACAGATACGGA | GGAAGCCAAGACTTCACACTG |
| **MIP-1α** | TCCCAGCCAGGTGTCATTTT | TTGGAGTCAGCGCAGATCTG |
| **MSR1** | TGAACGAGAGGATGCTGACTG | GGAGGGGCCATTTTTAGTGC |
| **PGC-1α** | TGATGTGAATGACTTGGATACAGACA | GCTCATTGTTGTACTGGTTGGATATG |
| **PGC-1β** | GGCAGGTTCAACCCCGA | CTTGCTAACATCACAGAGGATATCTTG |
| **TBP** | GGCCTCTCAGAAGCATCACTA | GCCAAGCCCTGAGCATAA |
| **TGFβ** | CAACCCAGGTCCTTCCTAAA | GGAGAGCCCTGGATACCAAC |
| **TNFα** | CACAAGATGCTGGGACAGTGA | TCCTTGATGGTGGTGCATGA |

**Suppl. Table 1** ***RT-PCR primers.***

# 4 Discussion

In the following section, general issues of this project, considerations that were beyond the scope of either manuscript, and a comparison between the *in vitro* and the *in vivo* model will be discussed. The first manuscript "The PGC-1 coactivators repress transcriptional activity of NF-κB in skeletal muscle cells" will be referred to as Ms1, the second manuscript "The PGC-1 coactivators promote M2 polarization of tissue macrophages in skeletal muscle" will be referred to as Ms2.

## 4.1 General discussion

The work presented here explored the relationship between PGC-1 coactivators and inflammatory reactions in skeletal muscle. The project was undertaken in order to clarify whether PGC-1α and PGC-1β have anti-inflammatory properties, a question that had not been resolved previously. Using a C2C12 cell culture model and adenoviral PGC-1 overexpression, this question was first addressed *in vitro*. In this system both PGC-1α and PGC-1β had repressive effects on pro-inflammatory gene expression elicited by TNFα, different TLR agonists and saturated FFAs. The repression was however target gene- and stimulus-specific arguing against a general immunosuppressive function of PGC-1 coactivators but rather for a differential fine-tuning of inflammatory reactions. The observation that PGC-1 coactivators are indeed able to reduce pro-inflammatory cytokine expression positions them at a major crossroad of two relevant physiological areas, namely, metabolism and inflammation. Innumerous studies in recent years have uncovered manifold interactions between those two areas and more importantly, their mutual dysregulation in many diseases. It is therefore not too surprising that a versatile, metabolic regulator like PGC-1 also plays a role in immune function, which however had not been proven so far. The anti-inflammatory effects of PGC-1s are also consistent with the beneficial effects of exercise involving the attenuation of chronic systemic inflammation (375).

In an attempt to elucidate how PGC-1 coactivators influence inflammatory reactions, stimulation with TNFα was analyzed in greater detail. Computational predictions pointed to the NF-κB pathway as main target of PGC-1´s repressive action. Analysis of the components in this pathway showed that both PGC-1α and PGC-1β interfered with an activating phosphorylation of the NF-κB family member p65. Lack of phosphorylation then leads to incomplete activation of transcription, explaining the lowered pro-inflammatory cytokine levels. As PGC-1α and PGC-1β have been described as coactivators so far, a direct corepressor function was unlikely and would have been hard to reconcile with their structure including an AD. The modulation of PTMs of members of the NF-κB pathway is therefore a more reasonable, indirect model. The ability to affect p65 phosphorylation

furthermore confirms the specific influence of PGC-1 coactivators on inflammatory reactions as opposed to a general immunosuppressive role. More precisely, PGC-1 coactivators do not repress transcription driven by NF-κB dimers that lack p65. In this way, they shape the response of muscle cells to inflammatory stimuli without completely repressing it. As p65 is responsible for many of the detrimental effects brought about by chronic inflammation, PGC-1 coactivators may act protective in this regard. Moreover, PGC-1α but not PGC-1β induced several components of the noncanonical NF-κB signalling pathway, which might explain differences between the two coactivators and further supports the concept that PGC-1s shape NF-κB-driven inflammatory responses. As the function of noncanonical NF-κB signalling in skeletal muscle is poorly understood, the consequences of this altered signalling remain to be determined. Recently, oxidative muscle metabolism was associated with activation of noncanonical NF-κB signalling, which fits well with the known roles of PGC-1 coactivators as potent inducers of oxidative pathways (359).

The p65 phosphorylation deficit has several causes: On the kinase side, insufficient Akt phosphorylation presumably fails to activate IKKα which in turn limits the scale of p65 phosphorylation. Concerning phosphatases, an involvement is likely as short-term TNFα stimulation did not yield overt differences in p65 phosphorylation as observed at later time points. Unfortunately, we were not able to identify the corresponding enzyme(s), but can exclude that PP1 and PP2A play a role. It would be of great interest to pinpoint that phosphatase as it might have a broader effect on other phosphorylation-sensitive processes in the cell (for further discussion of that point see 4.2.2). Induction of such a phosphatase would also be a good model to explain repressive effects of coactivator proteins as their primary function would be transcriptional initiation within the phosphatase promoter in accordance with their known molecular mode of action. Only the secondary effect would then be a repression as the phosphatase removes activating phospho residues. It is even possible, that the same phosphatase that dephosphorylates p65 also dephosphorylates Akt and therefore negatively affects its activity.

A second mechanism operating in conjunction with decreased phosphorylation to diminish pro-inflammatory cytokine expression is transrepression by PPARα. PGC-1α and PGC-1β both considerably increase expression of PPARα while inhibition of this nuclear receptor normalizes cytokine production at least for PGC-1β. Several subforms of transrepression have been described involving direct binding of nuclear receptors to NF-κB subunits as well as competitive binding to coactivators without direct contact. PPARα could potentially associates with p65 directly when bound to κB sites which might sterically block access to serine residues within p65 and thus hamper phosphorylation. If a sumoylation-dependent mechanism plays a role as previously shown for PPARγ (278) remains an interesting open question. Anti-inflammatory properties have been ascribed to many nuclear receptors including PPARα, underlining the feasibility of this result. Furthermore, fibrate

drugs that target PPARα and are widely used to lower cholesterol and triglyceride levels in humans, have been linked to anti-inflammatory action in most clinical studies on that issue (379).

In nonstimulated cells, PGC-1β but nor PGC-1α further repressed expression of p65 and p50, and consequently, DNA binding of these NF-κB family members representing another example of differential regulation between both coactivators. The repressive effect of PGC-1β as opposed to PGC-1α on cytokine expression in the basal state can be attributed to this property. As PGC-1β is not induced upon exercise in skeletal muscle and its function in this organ remains obscure, the physiological meaning of this observation is not immediately evident. The fact that skeletal muscle does however express considerable amounts of PGC-1β suggest some relevance of this factor. One putative role could be the prevention of inflammation as inflammatory reactions accompany many musculoskeletal disorders and deteriorate muscle function.

To our knowledge, this is the first report investigating the impact and mechanistic basis of PGC-1α and PGC-1β in cultured skeletal muscle cells regarding inflammatory responses. As NF-κB signalling also negatively acts on PGC-1 coactivator levels (at least in the long run, see 4.2.1 for further discussion), and thus on oxidative metabolism, these two pathways are more closely intertwined than supposed so far. In the context of metabolic disorders that are accompanied by low levels of both coactivators in skeletal muscle and a persistent, subclinical inflammation, these findings strongly suggest a causality between dysregulated PGC-1 coactivator expression and elevated inflammatory markers. Inversely, healthy muscles expressing sufficient amounts of PGC-1α and PGC-1β are protected from inflammatory insults to a certain degree. As chronic inflammation is detrimental and accelerates the progression of many diseases, preventing the decline of PGC-1α and PGC-1β in skeletal muscle during the course of such diseases appears as an attractive strategy to improve overall health. Reducing these diseases only to the antagonism of PGC-1 coactivators and NF-κB in skeletal muscle is however certainly an oversimplification and many other factors need to betaken into account to understand the pathology. The *in vitro* model that was used here however resolves basic mechanisms of this antagonistic action that are less evident in more complex systems for example diabetic human patients due to the multitude of confounding factors.

In an attempt to replicate the data *in vivo*, we encountered that PGC-1α and PGC-1β expression in skeletal muscle influenced macrophage polarization within the tissue. Even though pro-inflammatory gene expression was not changed in this model during an inflammatory response compared to WT animals, a higher number of alternatively activated macrophages and a cytokine profile associated with this state prevailed in the presence of PGC-1 coactivators. Therefore, PGC-1 coactivators exerted an anti-inflammatory role also *in vivo*, however in an unexpected indirect way. Most likely, this skewing of macrophages towards alternative activation was mediated by low IL-12 levels derived directly from muscle cells while high M2 cytokine expression probably originated from the activated macrophages themselves.

An influence of PGC-1α or PGC-1β expression in any tissue on immune cells has not been demonstrated before but this observation again shows how immune and metabolic pathways are intricately linked within and between cells. The involvement of the immune system in obesity and its sequelae is well characterized and classically activated macrophages foster the chronic inflammation and thus contribute to deterioration of patients´ health. In contrast, alternative macrophage activation confines inflammation and may even act protective. Although there are major gaps in our knowledge on macrophage polarization, its regulation and function in skeletal muscle, our findings provide another jigsaw piece to understand mutual influences of immune and muscle cells under conditions of inflammation. As this crosstalk is harmful in chronic diseases, the results presented here underline the importance of considering an integrated picture of muscle not only as metabolic but also as immune organ. The potential of PGC-1 coactivators to influence macrophage polarization therefore adds another layer of complexity to the multiple functions of these coactivators in skeletal muscle and represents an elegant, indirect way of anti-inflammatory action. Targeting PGC-1 coactivators could therefore be a valid strategy in metabolic as well as in skeletal muscle disorders that are accompanied by detrimental, inflammatory reactions.

## 4.2 The PGC-1 coactivators repress transcriptional activity of NF-κB in skeletal muscle cells

### 4.2.1 Inflammatory stimuli and their influence on PGC-1 coactivators in skeletal muscle cells

The chronic, low-grade inflammation that accompanies obesity is characterized by elevated levels of FFAs and pro-inflammatory cytokines including TNFα and IL-6. To evaluate the effect of PGC-1 coactivators on inflammation in our C2C12 model, we applied different inflammatory stimuli to elicit an immune response evident from the production of inflammatory cytokines. We found that TNFα, TLR1/2, TLR4 and TLR6/2 agonists as well as saturated fatty acids induced IL-6 and TNFα expression and transcription from an NF-κB reporter in this model in accordance with previous reports (Ms1, Fig. 1, Fig. 2, Fig. 3, Suppl. Fig 1C and 1D) (380-383). Besides TNFα, it seemed obvious to also employ IL-6 in this regard, as both cytokines are commonly linked to the development of metabolic diseases. In contrast to TNFα, IL-6 failed to elicit pro-inflammatory cytokine expression (Fig. 9A). IL-6 binds to the IL-6 receptor (IL-6R) that signals in conjunction with gp130. Hyper-IL-6 is a fusion protein of IL-6 and the soluble IL-6 receptor that acts on cells expressing gp130 but insufficient amounts of membrane-bound IL-6R (384). Its bioactivity was shown to be 100-1000 fold higher than unlinked IL-6/IL-6R on hematopoietic progenitor cell proliferation, but treatment of

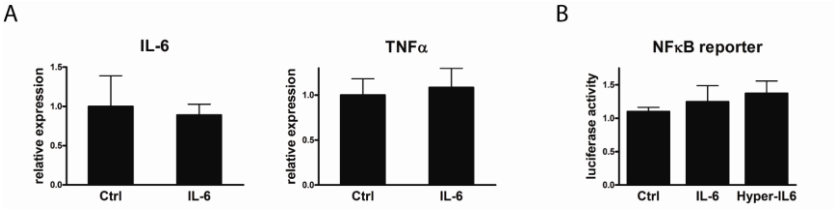



**Figure 9** ***IL-6 does not elicit transcription of inflammatory markers*****. A,** C2C12 cells were differentiated and treated with IL-6 for 2h. Expression of IL-6 and TNFα was assessed by real-time PCR. **B,** C2C12 cells were transfected with a NF−κB reporter construct (or a mutated reporter construct as control) and treated with IL-6 or Hyper-IL-6 (384) for 2h. Luciferase activity was determined and is expressed as ratio of wt to mutated reporter gene expression. Values represent the mean of at least 3 independent experiments ±SEM.

C2C12 cells with neither IL-6 nor Hyper-IL-6 yielded NF-κB activation (Fig. 9B) although these cells have been previously shown to express gp130 (385). This fits the notion that IL-6 is a potent inducer of the JAK/STAT and the MAPK/ERK pathway but not the NF-κB pathway (386). The expression of IL-6 itself is however under the control of NF-κB, JNK and PKC and possibly other pathways in skeletal muscle cells. Its effect on the muscle depends on the exposure time: While chronically elevated IL-6 levels, as present in obesity, impair insulin signalling, transient bouts of IL-6 enhance insulin sensitivity (385,387). This dual regulation also suits its role as a myokine in exercise. The

absence of pro-inflammatory cytokine expression (Fig. 9A) and the lack of NF-κB activation (Fig. 9B), which both are possibly harmful events in skeletal muscle, are therefore well in line with the beneficial effect attributed to short-term IL-6 exposure. As this study was focused on pro-inflammatory cytokine production and the NF-κB pathway, which were both unaffected by IL-6, this cytokine did not fulfil the criteria of an inflammatory stimulus in our model.

Expression from adenoviral vectors leads to a strong and persistent elevation of the respective mRNA and protein levels. Therefore, the *in vitro* study exhibited high differences in PGC-1α and PGC-1β expression between PGC-1 and GFP expressing cells that tended to be slightly induced by treatment with TNFα, albeit without reaching statistical significance (Ms1, Suppl. Fig. 1A and 1B). Endogenous levels of PGC-1 coactivator were repeatedly described to be affected by inflammatory reactions, although with inconsistent results. LPS-induced sepsis has been studied most extensively in this regard: In liver, heart and kidney, mRNA levels of PGC-1α, ERRα and MCAD were found to be markedly reduced 16h after LPS injection (388). The suppression of PGC-1α by LPS was further confirmed by another study in heart *in vivo* (389) and by treatment of cardiac cells with LPS or TNFα *in vitro* (390,391). PGC-1α levels could be rescued by inhibition of NF-κB indicating the involvement of this pathway. Reduced oxygen delivery and swollen mitochondria with impaired function are found in sepsis-associated acute kidney injury due to reduced PGC-1α levels that correlate with the degree of renal impairment (392). Along these lines, TNFα treatment of tubular cells for 48h reduced PGC-1α expression and oxygen consumption. This effect could be reversed by excess PGC-1α (392). Inversely, mice with a PGC-1α deletion had normal kidneys if unchallenged, but suffered from persistent injury after endotoxemia (392). In contrast to the findings of Kim et al. (388), PGC-1α and PGC-1β were both found to be induced in livers of septic mice in a TLR2-dependent manner (393). This induction further depended on the adaptor proteins TRAM and TRIF as well as on IRF3/7 as shown with deletion models (393). However, signalling of TLR2 through these alternative adaptors has not been confirmed by any other report.

In skeletal muscle, the situation is similar as both repression and induction of PGC-1 coactivators were observed. In an early study both, LPS and the pro-inflammatory cytokines TNFα and IL-1β elevated PGC-1α expression in cultured skeletal muscle cells *via* the MAPK/p38 pathway, leading to increased expression of genes in oxidative phosphorylation (106). More recently, sepsis was shown to decrease PGC-1α and PGC-1β in skeletal muscle of rats and mice 16h after LPS injection (394). These data are supported by a study in diaphragm, where LPS diminished fatty acid oxidation alongside a decrease in PGC-1α and PGC-1β at the same time-point (395). The differences may depend on the time-point chosen, as intraperitoneal injection of LPS induced PGC-1α in skeletal muscle after 2h but decreased its expression after 24h, arguing for a dual mode of action with a short-term up- but a long-term down-regulation (396). This model would also fit the induction of PGC-1α

after exercise in the presence of myokines that return to basal levels quickly whereas the low-grade inflammation in metabolic disorders impairs PGC-1α and PC-1β expression in skeletal muscle due to the chronic nature of the inflammatory mediators involved. Similar observations with palmitate back such a model, as long-term treatment (16h) of C2C12 cells repressed PGC-1α (but not PGC-1β) expression while at earlier time-points, PGC-1α was induced under these conditions (397). In accordance with our results, palmitate, but not oleate, elicited these effects that were furthermore dependent on MEK1/2 and NF-κB (397). In contrast to that, a study in vascular smooth muscle cells implicated oleic acid in a decrease and palmititc acid in an increase of PGC-1α levels after 24h (398).

Our cell culture model also supports a biphasic regulation of both PGC-1 coactivators by inflammatory stimuli as mRNA levels of both PGC-1α and PGC-1β tended to be increased by TNFα after 2h in cells expressing the transgene (Ms1, Suppl. Fig. 1A and 1B), which is also evident for PGC-1β on protein level (Ms1, Fig. 6A and Suppl. Fig. 4B). The effect is however absent in cells overexpressing GFP (Ms1, Suppl. Fig. 1A and 1B) suggesting that short-term TNFα treatment acts mainly on the transgene and not on the endogenous PGC-1 isoforms. To further elaborate on the effect of inflammatory stimuli on PGC-1 coactivator expression, two PGC-1α reporter constructs containing either the canonical or the alternative promoter were employed. The PGC-1α isoforms that derive from the alternative promoter (PGC-1α-b and PGC-1α-c) are upregulated by exercise and, in fact, they constitute the major share in contraction-induced PGC-1α in skeletal muscle (44). Their more dynamic expression raised the possibility that inflammatory stimuli might specifically act on those

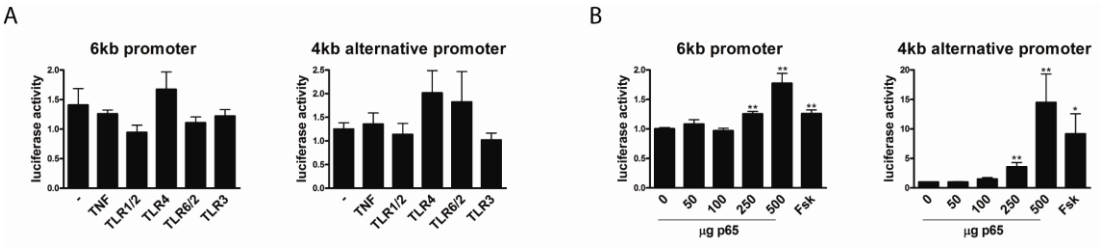



**Figure 10** ***Short-term inflammatory stimulation does not induce the PGC-1α promoter and p65 overexpression only at high levels*****. A,** C2C12 cells were transfected with a canonical (6kb promoter) or an alternative (4kb alternative promoter) reporter construct for PGC-1α. After 24h, cells were treated with TNFα or TLR agonists for 2h and luciferase activity determined. **B,** C2C12 cells were transfected with a canonical (6kb promoter) or an alternative (4kb alternative promoter) reporter construct for PGC-1α, increasing amounts of p65 or treated with forskolin (Fsk) for 4h as positive control and luciferase activity was determined. Values represent the mean of at least 3 independent experiments ±SEM. # $P<0.05$, treatment versus Ctrl.

isoforms in skeletal muscle as has been shown for β-adrenergic signalling previously (44). A 2h stimulation with TNFα or different TLR agonists (at the concentration used for all experiments) did not yield a significant induction of either construct (Fig. 10A) in accordance with results from GFP overexpressing cells. Cotransfection of high levels of p65 did however induce the canonical promoter slightly and the alternative promoter more strongly (Fig. 10B) confirming that the alternative promoter

is subject to a more variable regulation with higher plasticity than the canonical promoter. These results illustrate that regulation of PGC-1α by inflammatory stimuli is complex and not only depends on the duration of the stimulus but also the concentration. The differential effect on the canonical and the alternative isoform further increases the intricacy and may explain why previous reports varied considerably in their results. As the alternative PGC-1α isoform only accounts for a fraction of total PGC-1α amounts in muscle cells, the functional significance of this increase warrants further investigation. Biologically, an acute induction of PGC-1α (and presumably PGC-1β) elicited by high levels of inflammatory mediators could be a means of ensuring an adequate energy supply under conditions of infection where the muscle cell needs to fight pathogens which requires an additional amount of transcription, translation, secretion etc.

Opposed to acute, high levels of inflammatory mediators, their chronic presence decreased PGC-1 coactivator levels in the biphasic model. Examining long-term (48h) stimulation in our model, TNFα tended to decrease both coactivators (Fig. 11A), while TLR1/2 and TLR4 agonists did not have an influence on PGC-1β levels (Fig. 11B). TLR6/2 agonists tended to decrease PGC-1β and all 3 significantly lowered PGC-1α levels (Fig. 11B) supporting the biphasic regulation of PGC-1

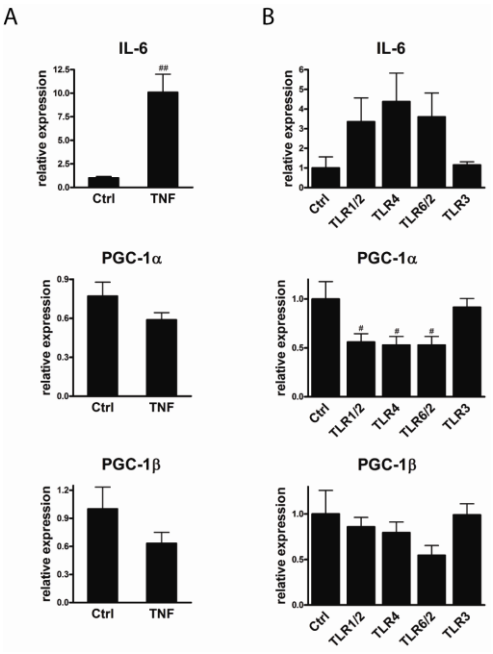



**Figure 11** ***Long-term inflammatory stimulation diminishes PGC-1 coactivators*****. A,** C2C12 cells were differentiated and treated with TNFα for 48h. Expression of IL-6, PGC-1α, and PGC-1β was assessed by real-time PCR. **B,** C2C12 cells were differentiated and treated with TLR agonists for 48h. Expression of IL-6, PGC-1α, and PGC-1β was assessed by real-time PCR. Values represent the mean of at least 3 independent experiments ±SEM. # $P<0.05$, treatment versus Ctrl.

coactivators. Treatment of C2C12 cells with fatty acids for 48h was not possible as the medium used was devoid of serum to exclude endotoxin contaminations not allowing for cell survival for such long periods of time. The repression of PGC-1α and PGC-1β at least by some chronically applied inflammatory stimuli fits in well with the dysregulation of these coactivators in skeletal muscle of obese patients that also suffer from a chronic inflammation, and it proposes that chronically elevated cytokine levels are causal to this dysregulation.

Furthermore, chronic obstructive pulmonary disease (COPD) is frequently associated with peripheral muscle wasting, and patients exhibit high TNFα levels in skeletal muscle with decreased oxidative capacity (399). In these muscles, PGC-1α is diminished and this suppression can be recapitulated by chronic treatment (>24h) of C2C12 cells with TNFα (399,400). TNFα-induced inhibition of oxidative phosphorylation is NF-κB dependent (399) and according to their function, more evident in oxidative than in glycolytic muscle fibers if mice are exposed to cigarette smoke (400). Interestingly, blood levels of TNFα negatively correlate with PPARα expression in skeletal muscle of COPD patients that show low PGC-1α levels (401).

Our data, the examples of sepsis (at least the majority of published data) and COPD therefore establish a negative regulation of oxidative metabolism including its master regulators, PGC-1α, by chronic inflammatory signals in skeletal muscle. Furthermore, we showed that at least *in vitro*, PGC-1 coactivators suppress pro-inflammatory cytokine expression in C2C12 cells by targeting NF-κB phosphorylation. The NF-κB pathway thus appears to be a nodal point between two fundamental biological programs, namely inflammation and oxidative metabolism which are subject to reciprocal regulation by one another. This notion may extend to other organs especially those with high oxidative capacity and to inflammatory diseases that are often accompanied by mitochondrial dysfunction.

From an evolutionary perspective, this mutual regulation is not so surprising as metabolic and immune functions have developed from a common ancestral structure, i.e. the fat body in *Drosophila* (402). This organ comprises homologues of liver, adipose tissue and immune system and functions to sense nutrient and energy status but also pathogen presence. These cues are then translated into an appropriate metabolic or immune response to assure survival. In mammals, these tasks are divided between specialized organs that however still carry their developmental heritage, a putative common regulatory network of metabolic and immune signalling. Importantly, both liver and adipose tissue still contain large numbers of immune cells (Kupffer cells and ATMs) enabling constant interaction and an integration of metabolic and immune signals (402).

### 4.2.2 Regulation of other NF-κB pathway modulators by PGC-1 coactivators

In an attempt to delineate the mechanism of PGC-1-mediated downregulation of inflammatory genes, we identified the NF-κB pathway as crucial player and more specifically, decreased phosphorylation of p65. To answer the obvious question about the responsible kinase and/or phosphatase, we assessed the levels of different kinases able to change p65 phosphorylation. Among these, MSK1 and PKAc directly target p65 but their levels were either unchanged or even induced in the presence of PGC-1α and PGC-1β (Fig. 12A and 12B). Although kinase activities are often regulated at the posttranscriptional level, these results did not imply an involvement of these two kinases in

downregulating p65 activity. We further analysed levels of p38 and activity of Akt, both kinases that do not directly target p65 but modulate MSK1 and IKKα activity, respectively. While p38 levels were unchanged (Fig. 12A and 12B), Akt was markedly decreased (Ms1, Fig. 7A). We therefore postulate a mechanism of PGC-1-dependent repression of Akt that acts on IKKα which subsequently is unable to phosphorylate p65 sufficiently, ultimately resulting in diminished NF-κB activity.

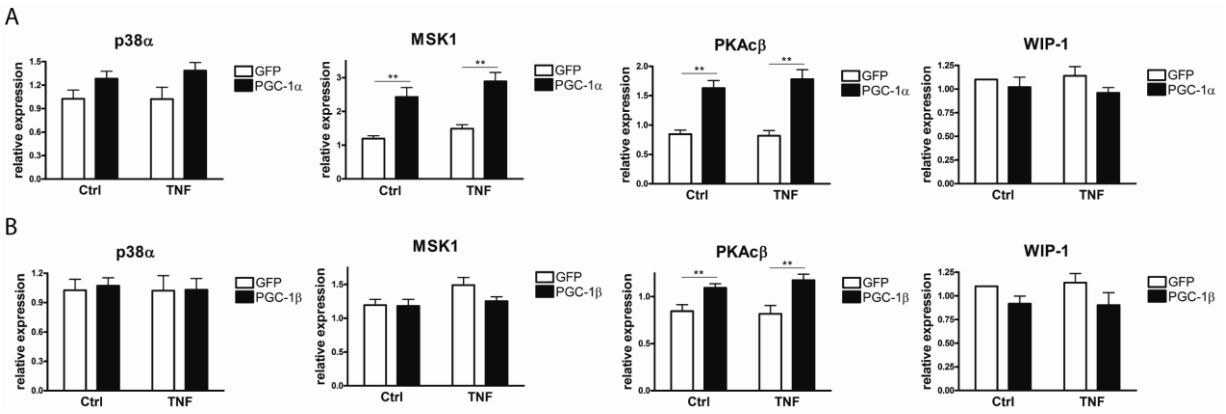



**Figure 12** ***p38, MSK1, PKAc and WIP-1 are not responsible for lower p65 phosphorylation*****. A, B,** Differentiated C2C12 myotubes overexpressing PGC-1α and GFP (Panel A) or PGC-1β and GFP (Panel B) were treated with TNFα for 2h. Expression of the p65 kinases p38α, MSK1, PKAcβ and the phosphatase WIP-1 was determined by real-time PCR. Values represent the mean of at least 3 independent experiments ±SEM. ** $P<0.01$ PGC-1α/β versus GFP.

As we observed similar levels of p65 phosphorylation between conditions after 5min of TNFα treatment (compared to reduced levels after 2h in the presence of PGC-1α/β, Ms1, Fig. 7B and 7C), we also considered phosphatase involvement. The only known p65-specific enzyme, WIP-1, did not change expression (Fig. 12A and 12B) and is therefore unlikely to be important in the phenomenon observed. Inhibition of the broad target phosphatases PP1 and PP2A that are known to also dephosphorylate p65 yielded the expected stabilization of p65 phosphorylation. The effect of lower p65 phosphorylation levels in the presence of PGC-1α/β was however still present albeit at substantially higher levels (Ms1, Fig. 7D, 7E and 7F). PP2A and PP1 are therefore also most likely not involved in these processes. Interestingly, okadaic acid (the PP1/PP2A inhibitor) also enhanced PGC-1α levels (Ms1, Fig. 7D and 7E). So far, an effect of okadaic acid on PGC-1α has only been described with regard to localization: hyperphosphorylated PGC-1α moves out of inactive nuclear speckles, a feature common to classic SR proteins that are involved in pre-mRNA splicing (41). Investigating which serine and/or threonine residues within PGC-1α are targeted by PP2A and PP1 would broaden our knowledge on the regulation of PTMs and might open up new ways to influence PGC-1α protein stability (see 1.1.3).

In addition to phosphorylation of p65, multiple signals are able to modulate NF-κB activation and impinge on signalling (see 1.2.2). Bcl3 is an atypical IκB (see 1.2.1) and was shown to interact

with PGC-1α in order to synergistically coactivate ERRα and PPARα in cardiac cells (403). This interaction led us to determine whether Bcl3 might be implicated in the repression of inflammatory NF-κB signalling in our model. However, Bcl3 mRNA levels (Fig. 13A and 13B) followed the expression pattern of pro-inflammatory cytokines (Ms1, Fig. 1A and 1B) rendering it a likely downstream target of NF-κB, but not an upstream regulator. The same holds true for the dual ubiquitin editing enzyme A20 (Fig. 13A and 13B), and indeed a negative feedback loop has been known for a long time (404). Higher A20 levels that would decrease pro-inflammatory cytokine expression would also change DNA binding of the p65/p50 heterodimer. Therefore, these data fit results from the TransAM where no such difference could be detected. An induction of IκBα as previously described in quadriceps of whole body PGC-1α transgenic mice (405) would also influence p65/p50 DNA binding. We could not recapitulate this induction in our *in vitro* system in line with TransAM results.

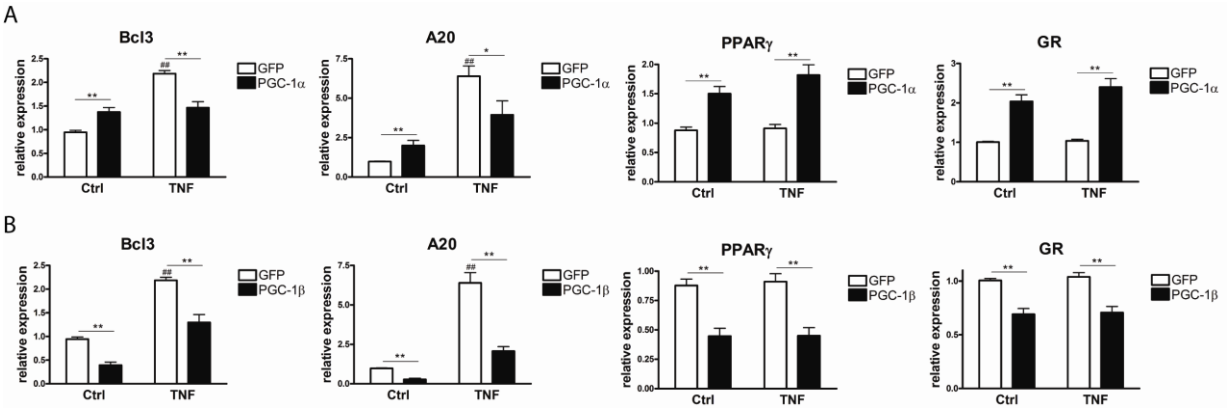



**Figure 13** ***Bcl3, A20, PPARγ and GR do not contribute to a common PGC-1α/β-mediated mechanism of NF-κB repression*****. A, B,** Differentiated C2C12 myotubes overexpressing PGC-1α and GFP (Panel A) or PGC-1β and GFP (Panel B) were treated with TNFα for 2h. Expression of Bcl3, A20, PPARγ and GR was determined by real-time PCR. Values represent the mean of at least 3 independent experiments ±SEM. # $P<0.05$, ## $P<0.01$ GFP TNF versus GFP Ctrl, * $P<0.05$, ** $P<0.01$ PGC-1α/β versus GFP.

As transrepression is a mechanism that does generally not change DNA binding of the targeted transcription factor, we also investigated nuclear receptors that had previously been shown to mediate transrepression. We focused on PPARα, PPARγ and GR that are furthermore coactivated by PGC-1α. PPARα, that is also coactivated by PGC-1β, was strongly induced in the presence of both PGC-1 coactivators (Ms1, Fig. 8A and 8B), while PPARγ and GR were induced by PGC-1α but reduced by PGC-1β (Fig. 13A and 13B). The opposite regulation of PPARγ and GR precludes a uniform transrepression mechanism mediated by these coactivators, even though PPARγ and GR might contribute to transrepression in the presence of PGC-1α. An *in vivo* study on inflammatory bowl disease found indeed amelioration of colitis by treatment with conjugated linoleic acid that was mediated by PPARγ. (406). Treated mice also exhibited higher PGC-1α levels while NF-κB activation and TNFα expression were repressed reminiscent of the situation in our model. Unfortunately,

confirming the large increase of PPARα on protein levels was impossible due to inferior quality of commercially available antibodies.

### 4.2.3 Direct interaction of PGC-1 coactivators and NF-κB subunits

An inhibitory effect may stem from a direct protein-protein interaction that depending on the function of the inhibited protein either hinders it from DNA binding, or interaction with other proteins, or blocks its enzymatic activity. Therefore, a direct interaction of PGC-1α and PGC-1β with p65 and/or p50 was a possible inhibitory mechanism which could have diminished pro-inflammatory cytokine production. In bone marrow stromal cells constitutive, direct binding of PGC-1α to p65 and p50 was reported which decreased in the presence of PPARγ ligands (407). The authors therefore hypothesized that NF-κB and PPARγ compete for the shared coactivator PGC-1α. Its recruitment to PPARγ would limit NF-κB-dependent IL-6 expression and consequently activation of multiple myeloma cells in their model (407).

In hepatocytes, p50 and PGC-1α were shown to interact upon treatment with FFAs regardless of their saturation status (408). Both saturated and unsaturated FFAs further led to binding of this complex to the IL-10 promoter. Complex formation and IL-10 expression could be abolished by inhibition of NF-κB. However, inhibition of PGC-1α by antisense oligonucleotides in liver resulted in a significant increase in IL-10 expression (408) which would render PGC-1α a transcriptional corepressor of p50 on the IL-10 promoter. The domain structure of PGC-1α that enables recruitment of cofactors for initiation complex formation (see 1.1.1) and the multitude of coactivation events published, yet rather argue against such a function.

The most careful characterization of a PGC-1α-NF-kB interaction was accomplished in cardiomyocytes (409). By means of EMSA and co-immunoprecipitation, an interaction of PGC-1α and p65 was observed that increased upon TNFα treatment and involved the LXXLL motifs in the PGC-1α N-terminus. On the basis of this interaction the authors propose a model of direct PGC-1α inhibition by p65 which leads to lower PDK4 levels and subsequently to a higher glucose oxidation rate (409). However, siRNA directed against PGC-1α did not change PDK4 expression nor were NF-κB target genes like IL-6 and MCP-1 affected by siRNA directed against p65 and PCG-1α. The functional consequences of this interaction are therefore not fully clear and the study did not comprise any ChIP experiments.

In skeletal muscle cells, no direct interaction between a NF-κB family member and either PGC-1α or PGC-1β has been found so far. If an interaction takes place, we argued that at least in our model, PGC-1α/β has to remain bound to p65 on κB sites, as DNA binding of p65 was not changed in

PGC-1α/β overexpressing cells after TNFα treatment despite diminished cytokine expression (Ms1, Fig. 5D and 5E). To test this possibility, we performed chromatin immunoprecipitation (ChIP) in PGC-1α overexpressing cells and evaluated the abundance of PGC-1α on κB sites in the IL-6 and TNFα promoter. We could not detect any PGC-1α present at these sites whereas a clear enrichment was found in the MCAD promoter, a known PGC-1α target gene (Fig. 14). The lack of a specific, high affinity PGC-1β antibody prevented us from assessing the same parameters in PGC-1β overexpressing cells. The ChIP data for PGC-1α however do not support a model with a direct PGC-1-p65 interaction at the promoters of inflammatory p65 target genes and thus rather point to an indirect mechanism. Direct binding to p65 at the DNA with negative effects on gene expression (as observed for IL-6 and TNFα for example) would again put PGC-1 in the role of a corepressor. Although not completely

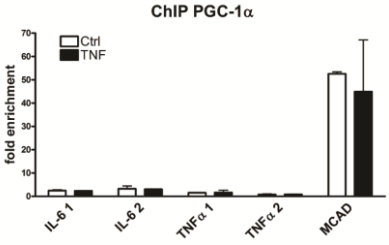



**Figure 14** ***PGC-1α does not bind to the promoters of IL-6 and TNFα.*** Differentiated C2C12 myotubes overexpressing PGC-1α were treated with TNFα for 2h. DNA fragments enriched after crosslinking, sonication, and subsequent ChIP against flag-tagged PGC-1α were analyzed by real-time PCR and values are expressed as fold enrichment over the intronic region of TBP (a non-target region of PGC-1α). IL-6 1, IL-6 2, TNFα 1 and TNFα 2 denote experimentally validated κB sites in the IL-6 and the TNFα promoter respectively. MCAD stands for the ERRα binding site in the MCAD promoter (a known PGC-1α target region).

excluded, it would be difficult to reconcile such a corepressor function with the well-established coactivator role of PGC-1α and PGC-1β, which both effectively tether proteins to the promoter that activate transcription. Also from a conceptual point of view, an indirect mechanism with an intermediate step in which PGC-1 coactivators induce expression of a factor that inhibits NF-κB activity is therefore the more likely model. Our data suggest that this factor is PPARα which is induced by both PGC-1α and PGC-1β, and is capable of acting transrepressive on NF-κB. Furthermore, the involvement of a p65 phosphatase (different from the known p65 phsophatases PP2A, PP1 and WIP1) is likely.

## 4.3 The PGC-1 coactivators promote M2 polarization of tissue macrophages in skeletal muscle

### 4.3.1 Anaesthesia and PGC-1 transgene expression in skeletal muscle

As isoflurane anaesthesia is a standard method to etherize mice and has been administered in our lab previously, the protocol for our *in vivo* experiments first comprised this form of inhalation anaesthesia to be able to inject mice *i.m.* The first batch of mice investigated for their response to TNFα and LPS were MCKα and WT animals that were sacrificed 4h post injection. At the mRNA levels, we observed a complete loss of PGC-1α transgene expression in TA which we first ascribed to the inflammatory agents injected. Further experiments without injection proved this theory false but isoflurane anaesthesia alone elicited the same effect (Fig. 15A). We therefore had to adjust the anaesthetic, and tested the related substance sevoflurane. Anaesthesia induction was similarly fast compared to isoflurane, as was recovery. However, the repressive effect on transgenic PGC-1α expression after 4h was completely relieved (Fig. 15A). We also evaluated the expression of the PGC-1β transgene in MCKβ mice after anaesthesia with isoflurane and sevoflurane. Again, isoflurane

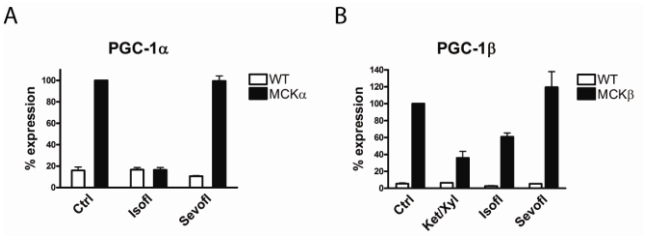



**Figure 15** ***Transgenic PGC-1 expression is sensitive to anaesthesia*** **A,** WT and MCKα mice were anaesthetized for 3 minutes by inhaling isoflurane (Isofl), sevoflurane (Sevofl) or left untreated (Ctrl). 4h post anaesthesia, TA muscles were analyzed for PGC-1α expression by real-time PCR. **B,** WT and MCKβ mice were anaesthetized for 3 minutes by inhaling isoflurane (Isofl), or sevoflurane (Sevofl), by intraperitoneal injection of a ketamine/xylazine mixture (Ket/Xyl) or left untreated (Ctrl). 4h post anaesthesia, TA muscles were analyzed for PGC-1β expression by real-time PCR. $n \geq 3$ animals per group.

reduced transgenic PGC-1β expression while sevoflurane did not interfere with mRNA levels (Fig. 15B). Additionally, we tested ketamine/xylazine injection as alternative anaesthesia method. Due to the different administration form, induction took longer, and more importantly, recovery was much delayed (hours versus minutes with both volatile anaesthetics). Besides not being justified regarding the relatively small intervention against the long duration of anaesthesia, this method depressed transgenic PGC-1β expression to an even greater extent than isoflurane inhalation. These data thus validate sevoflurane as anaesthetic of choice as it does not interfere with the transgene.

The depressive effect of isoflurane and ketamine/xylazine on transgenic PGC-1 expression was unexpected and no such interference has been described before. An important determinant in this

depression is the time of sacrifice which was rather short (4h post anaesthesia) as longer periods of recovery compensate the effect. The complete repression of transgenic PGC-1α by isoflurane versus the partial repression of transgenic PGC-1β can be explained by different levels of overexpression which are higher in MCKβ animals (about 15 fold versus 7 fold in MCKα mice, see Ms2, Fig. 1A and 1B). It is astonishing that isoflurane and sevoflurane differ so fundamentally in their effects on PGC-1 transgene expression despite sharing a similar chemical structure and very similar properties regarding anaesthesia. The elimination of sevoflurane in the first 2h after discontinuation of anaesthesia is however 1.6 fold faster than with isoflurane, possibly accounting for the difference between the two anaesthetics (410). Transgene expression in both models is under the control of the MCK promoter. Why this promoter is sensitive to isoflurane exposure is not known but a possible mechanism could involve calcium fluxes. While isoflurane perturbs calcium homeostasis in skeletal muscle (411), sevoflurane has less influence in this regard (412). As the MCK promoter contains MEF2 sites (413) and MEF2 is sensitive to intracellular calcium concentrations (414), PGC-1 transgene depression could be a consequence of inadequate calcium levels under isoflurane anaesthesia. This would also fit the notion that isoflurane is a strong but sevoflurane only a weak muscle relaxant.

### 4.3.2 PGC-1 coactivators and anti-inflammatory cytokines

Numerous studies dealing with inflammatory reactions in diverse contexts have described levels of pro-inflammatory cytokines like TNFα, IL-6 and IFNγ as these are the main drivers of inflammation. Cytokines however rarely act individually but are rather part of a complex cytokine network that involves pro- but also anti-inflammatory factors which may synergize or neutralize each other depending on stimulus, timing etc. We evaluated a range of cytokines that are known to be involved in M1 or M2 macrophage responses in our injection and downhill running experiments to gain insight into the cytokine network acting in the MCKα/β models. Surprisingly, we did not observe a difference in expression of the pro-inflammatory cytokines TNFα, IL-6, MIP-1α and MCP-1 after PBS, LPS or TNFα injection *i.m.* (Ms2, Fig. 3A). A recent study that also employed the MCKα model and LPS injection *i.p.* reported similar findings with unchanged TNFα and IL-6 levels in skeletal muscle of transgenic mice (415). Unaffected IL-10 levels in skeletal muscle in this study are further in accordance with our results as are the previously discussed systemic levels of IL-6 and TNFα (415). The authors did not evaluate IL-12 expression or levels of anti-inflammatory cytokines other than IL-10. Our report is therefore the first to show the effect of PGC-1 coactivators on IL-12 expression in skeletal muscle and in myocytes in culture. The diminished IL-12 levels in muscle are likely the cause of the observed M2 skewing of tissue macrophages accompanied by a changed cytokine expression profile.

In primary hepatocytes, PGC-1α induction after fasting, exercise, glucagon, or cAMP exposure has previously been associated with an increase in IL-1Ra and IL-15Rα (416). The induction of these anti-inflammatory cytokines was further dependent on PPARα (416). We also detected higher expression of IL-1Ra in sedentary MCKα animals and in untreated, cultured myocytes overexpressing PGC-1α, this difference was however lost with any kind of injection (even PBS, which also causes a microinjury), treatment or training. A direct effect of PGC-1α on IL-1Ra in naïve muscle cells is therefore likely (similar to the situation in hepatocytes). A major role for IL-1Ra in M2 macrophage skewing is however implausible. In MCKβ mice, IL 1Ra expression was augmented after PBS and LPS injection as well as in sedentary and exercised animals, but the effect was not recapitulated in C2C12 cells overexpressing PGC-1β suggesting an indirect effect in this case with IL-1Ra release from immune cells.

Yet another study in liver established a mechanistic link between mitochondrial biogenesis and anti-inflammatory gene expression *via* the enzyme heme oxygenase 1 (HO-1) (417). Induction of HO-1 after LPS and carbon monoxide treatment induced IL-10 and IL-1Ra mRNA and protein expression and at the same time nuclear abundance of the TFs MEF2, Nfe2l2, and NRF-2, which is involved in mitochondrial biogenesis. These TFs were shown to be recruited to the promoter regions of IL-10 and IL-1Ra as opposed to the promoter regions of pro-inflammatory cytokines which are regulated primarily by NF-κB (417). HO-1 is thus another nexus of metabolic and (anti-)inflammatory signalling. As HO-1 appears upstream of MEF2 and thus upstream of PGC-1α, a direct effect of PGC-1α on anti-inflammatory cytokine expression is unlikely matching our results.

Interestingly, the same study established that this mechanism is also active in the macrophage cell line J774.1 (417), which fits the concept of anti-inflammatory M2 activation with higher expression of IL-10 and IL-Ra, and a concomitant switch in metabolism from glycolytic to oxidative. PGC-1β was shown to be vital in the induction of this oxidative, anti-inflammatory macrophage phenotype (327) while PGC-1α levels were found to be elevated in macrophages that undergo M2-like changes during prolonged high fat feeding (418). Inversely, LPS exposure downregulated PGC-1α (and PPARα) in bone-marrow derived macrophages thus enabling ROS production, a feature characteristic of pro-inflammatory M1 macrophages, as antioxidative enzymes like glutathione peroxidise and catalase were diminished (419). The suppression of PGC-1α in these cells depended on IRAK-1, which is also critically involved in the induction of Nox-1, a key component of the NADPH oxidase complex (419). As HO-1, the enzyme that connected mitochondrial biogenesis and anti-inflammatory gene expression in hepatocytes and macrophages (416), is also able to counteract NADPH oxidase function at least in endothelial cells (420), HO-1 seems to play a prominent role in the determination of the macrophage phenotype that correlates with PGC-1 expression. In contrast to all the previously described results, one report claimed that PGC-1β is a target of the IFNγ/STAT1 axis (M1) and that PGC-1β together with ERRα induces mitochondrial ROS production (421).

Therefore, more research is needed to fully establish the role of PGC-1 coactivators in macrophage activation. The currently available studies do foster an involvement in M2 determination though. It has to be stressed in this context, that the MCKα/β model we employed only expressed the transgene in skeletal muscle and that all other cells including macrophages had a WT genotype. If skeletal muscle-specific overexpression of PGC-1α/β also influences the expression of these coactivators in macrophages remains to be determined. In the light of a skewing towards the M2 phenotype, it seems likely that this would be accompanied by higher PGC-1 expression levels.

## 4.4 Comparison of *in vitro* and *in vivo* models

The general idea behind both the *in vitro* and the *in vivo* approach was to test if the presence of PGC-1 coactivators in skeletal muscle would influence inflammatory reaction. Therefore, we employed cultured C2C12 myotubes overexpressing PGC-1α or PGC-1β from adenoviral constructs and skeletal muscle-specific transgenic mice, respectively. *In vitro*, both coactivators were strongly overexpressed: PGC-1α yielded about 30000 fold while PGC-1β reached about 1500 fold compared to control cells overexpressing GFP (Ms1, Suppl. Fig. 1A and 1B). *In vivo*, the rate of overexpression was more moderate, i.e. about 7 fold for PGC-1α and about 15 fold for PGC-1β compared to WT animals (Ms2, Fig. 1A and 1B). The profoundly higher rates *in vitro* are on the one hand due to the very low expression of PGC-1α and the low expression of PGC-1β in control cells whereas WT animals do express significant amounts of both coactivators. The *in vitro* model thus rather resembles a comparison between MCKα/MCKβ animals and the respective skeletal muscle-specific deletion models that lack PGC-1α or PGC-1β. On the other hand, adenoviral expression is driven by the very potent CMV promoter also leading to sustained overexpression while in the animal model, the tissue specific MCK promoter controls PGC-1 coactivator expression. The sensitivity of this promoter e.g. to suppression by specific anaesthetics is discussed above (see 4.2.1). Yet another difference between the *in vitro* and the *in vivo* model derives from the fact that recombinant adenovirus remains epichromosomal whereas the transgene *in vivo* was inserted into the host genome during the creation of the MCKα/MCKβ line. The position of this insertion is however not known and therefore abnormal expression of genes at the insertion site cannot be excluded.

Generally, viral overexpression may not seem a good idea in the context of inflammatory studies as the virus itself may induce inflammatory signalling in the host cell. As control cells overexpressed GFP from a viral vector as well, the bias of the infection should be removed by comparing these two conditions. We further evaluated non-infected cells in comparison to GFP-expressing cells and did not find major differences at least in the expression of genes we were interested in. Therefore, we assumed that adenoviral infection is a valid model especially in the context of cultured myocytes that are postmitotic and difficult to transfect rendering standard plasmid transfection techniques unfeasible. Another possibility would have been the generation of stable cell lines overexpressing PGC-1 coactivaotrs or the isolation of primary muscle cells from WT and transgenic mice respectively. However, transgene expression in primary cells declines after a few passages which turns this strategy prone to errors.

The big differences described above between PGC-1 expression in the *in vitro* and the *in vivo* model are likely also part of explanation for divergent results. While *in vitro*, we observed a repression of IL-6, TNFα and MIP-1α by PGC-1α and PGC-1β after treatment with TNFα, injection of TNFα into TA of transgenic and WT mice did not yield such a difference. Furthermore, the basal repression

exerted by PGC-1β on these cytokines *in vitro* was not replicated *in vivo*. Considering that the GFP control cells barely express PGC-1α and are thus similar to MKO mice, we might extend this thought to the expression of pro-inflammatory cytokines. Their levels are higher in skeletal muscle of MKO than WT mice (158). Envisioning these levels as baseline would probably bring the *in vitro* and *in vivo* results closer together, as these pro-inflammatory cytokines should be lower in MCKα mice (because they are not different from WT in our experiments and levels in MKO muscle are higher than in WT according to literature). For PGC-1β, no data on inflammatory markers in a deletion model are available. Taking this scenario and our *in vitro* results including mechanistic details into account, an anti-inflammatory role of both PGC-1 coactivators is manifest. However, only lowered levels of these coactivators as present in diseases like diabetes or in genetically engineered animals make this property become evident in the way that inflammation cannot be confined. In contrast, WT levels seem to be high enough to control excessive inflammation in muscle cells, precluding the formation of a difference. This observation leads to the interesting hypothesis that PGC-1 coactivators act in a "threshold mode", meaning that expression above a certain level is necessary to exert their biological function (but further induction is not beneficial), while a drop below this threshold is detrimental.

Besides mere differences in expression levels of PGC-1 coactivators, the complexity of skeletal muscle as an organ as opposed to a pure myocyte culture should not be neglected. In addition to myofibers, skeletal muscle also contains satellite cells and connective tissue like the endomysium, the perimysium, and the epimysium, lining fibers, bundles and the whole organ, respectively. Furthermore, blood and lymphatic vessels traverse the organ while γ motoneurons and sensory neurons extend into the muscle spindle. Finally, immune cells like tissue resident macrophages and dendritic cells within skeletal muscle monitor the immune status of the organ. The complex interplay of these different cells types ensures proper muscle function. It is therefore not too surprising that results from *in vitro* experiments with only myocytes present cannot be readily translated into the *in vivo* setting. Injection of inflammatory stimuli and also of PBS causes a microinjury not present when treating cells with cytokines or TLR antagonists. This alone would lead to activation of the immune system at the injured site. This is also a very likely cause for differences between the control groups in the injection and the downhill running experiment. While sedentary animals in the latter were completely untouched apart from the short acclimatization period, control animals in the former were subject to injection which brought about similar effects as injection of inflammatory agents albeit at mush lower intensity. The inflammatory agents injected then again acted both on myocytes and immune cells. As immune cells are specialized to react to infection and injury their contribution to the cytokine response we measured was likely high. It is therefore possible that suppression of IL-6, TNFα and MIP-1α within myocytes due to PGC-1 overexpression occurred but was overridden by the powerful immune response mounted by immune cells and could thus not be detected. As we did not deplete macrophages from muscle and analyzed lysates of the whole organ, the origin of cytokines could not be traced back.

## 4.5 Perspectives

The interface of metabolic and immunological research is an active area of investigation and multiple layers of crosstalk between both sides have just started to emerge and to be understood. In the present study we explored the effect and the mechanisms of PGC-1α and PGC-1β overexpression on inflammatory reactions *in vitro* and *in vivo* in skeletal muscle. We found that both coactivators act in an anti-inflammatory manner in muscle cells as they suppress the expression of pro-inflammatory cytokines and diminish phosphorylation of p65 upon stimulation with inflammatory agents. In this regard, it would be of great interest to identify the kinase(s) involved in p65 phosphorylation under these conditions as well as a putative phosphatase that decreases p65 phosphorylation in the presence of either coactivator. We hypothesized that IKKα downstream of Akt could engage in such a phosphorylation as Akt activation was decreased by PGC-1α/β. Reconstituting Akt activity e.g. by adenoviral co-overexpression would therefore reverse the effect as would activation if IKKα. Experimentally validating these assumptions would support the mechanism by which PGC-1 coactivators decrease pro-inflammatory gene expression. A phosphatase screen could also help to identify a putatively new p65 phosphatase which is induced by PGC-1α/β and removes the TNFα-induced phospho-residue from p65 quickly.

Concerning PPARα-mediated transrepression, characterizing the complex in detail that is tethered to/kept at the p50/p65 dimer bound to DNA, would also broaden our understanding of anti-inflammatory actions exerted by nuclear receptors. The role of SUMOylation in this respect would merit special attention as an elegant study on PPARγ revealed its important function in the repression of NF-κB-dependent genes (278). Investigating mechanisms downstream of stimuli other than TNFα (like TLR agonists or FFAs) would further complement the present work.

*In vivo*, skewing of M2 macrophages by skeletal muscle PGC-1 is a very interesting and unexpected finding, some questions however remain unresolved to date. Primarily, determining the factor(s) responsible would be a major contribution to understand the complex interplay between muscle and immune cells under pro-inflammatory conditions. As we suspect diminished muscle IL-12 levels in transgenic mice to play an important role in this process, an illustrative experiment would be the reconstitution of IL-12. Several strategies would be possible: First, cross-breeding of MCKα/MCKβ mice with skeletal muscle-specific IL-12 transgenic mice would constitute a genetic model that according to our hypothesis would reverse M2 macrophage activation after inflammatory stimulation. Second, and less time-consuming, electroporation of TA with IL-12 plasmid DNA (422) would give the same result. As not all fibers are hit by this procedure, the effect would presumably be more subtle or maybe even insufficient to prevent M2 polarization. Third, exogenous IL-12 administration *i.m.* or *i.p.* could be employed to reverse the phenotype. If the low expression of IL-12 has a systemic effect in our model still remains to be clarified.

To follow up on the mechanisms and the factors mediating preferential M2 polarization upon inflammatory stimulation in transgenic mice, a cell culture model would complete the current study. Therefore, conditioned medium either from primary myocytes of MCKα/MCKβ mice or from C2C12 cells overexpressing PGC-1α/β could be transferred onto primary macrophages or an immortalized macrophage cell line and their polarization status monitored. Stronger M2 polarization as compared to macrophages exposed to control medium would be expected. Another advantage of this technique would be the possibility to analyse the conditioned medium for factors it contains. Of course, IL-12 would be an obvious candidate to look for, but a complete secretome analysis would be a more unbiased approach.

Isolating macrophages from skeletal muscle of our model would further facilitate to characterize these cells more comprehensively e.g. by flow cytometry. Analysis of the cytokines that were higher in whole muscle lysates but not in the C2C12 model like CCL1 for example, would confirm that they derive from immune and not muscle cells and that changes in their expression are thus secondary effects. Inversely, depletion of macrophages from skeletal muscle (312) would be another interesting tool to dissect which cues originate from muscle and which from immune cells.

As PGC-1 levels in skeletal muscle of obese and diabetic patients are dysregulated, exposing mice to a high fat diet (HFD) which evokes a systemic, low-grade inflammation instead of the more acute inflammatory stimulation with LPS and TNFα injection or a single bout of downhill running would be of prime interest in the long term. Investigating macrophage populations in skeletal muscle under these conditions in the presence of PGC-1 would validate the claim that restored PGC-1 levels not only improve metabolic but also inflammatory components in the metabolic syndrome by preventing pro-inflammatory M1 macrophage activation. The complex phenotype of the MCKα model on HFD regarding glucose homeostasis however has to be taken into account. Reciprocally, studies on macrophages in skeletal muscle-specific deletion models of PGC-1α and PGC-1β would complement this work. Higher mRNA levels of CD68 in skeletal muscle of MKO mice (158) already point towards a higher degree of M1 polarization supporting our results. A more careful analysis still warrants further investigation.

Elucidating basic mechanisms of direct PGC-1 coactivator action in muscle cells and their crosstalk to cells of the immune system is an important step in understanding the role of these cofactors in health and diseases. This knowledge is also indispensable to be able to develop strategies aimed at combating the consequences of dysregulation of PGC-1α and PGC-1β. Applying our findings to benefit patients suffering from the metabolic syndrome but maybe also from skeletal muscle disorders that involve an immune component would imply the availability of a specific PGC-1 agonist as genetic approaches are not yet in a safe stage regarding treatment of humans. Such an agonist has however not been found to date and intensive research in this direction is needed to translate our basic findings into a therapy. Our work shows that PGC-1α and PGC-1β are worthwhile targets that deserve the attention of future investigations to finally improve patients´ health.

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# 6 Curriculum vitae

## Personal details

| | |
|---|---|
| Name | **Petra Sabine EISELE** |
| Contact | Ahornstr. 30, CH-4055 Basel,<br>+ 41 (0) 61 554 31 83, petra.eisele@unibas.ch |
| Date of Birth | January 22, 1981 |
| Nationality | German |

## Education

| | |
|---|---|
| 2007 - 2012 | **PhD in Integrative Molecular Medicine**<br>Life Science Zurich Graduate School<br>University of Zurich and Biozentrum Basel, Switzerland<br>PhD thesis "The Role of PGC-1α and PGC-1β during Inflammatory Processes in Skeletal Muscle" |
| 2000 - 2006 | **Diploma in Biology (Dipl.-Biol.)**<br>University of Konstanz, Germany<br>Diploma thesis in Immunology: "Functional Analysis of the C-terminal Cytoplasmatic Tail of Chemokine Receptor CCR7" (awarded the VEUK prize for an outstanding graduation) |
| 2004 | **Exchange semester**<br>Department of Medical and Molecular Biosciences<br>University of Technology, Sydney, Australia<br>Supported by a Baden-Württemberg fellowship for highly qualified students |
| 1991 - 2000 | **Abitur**<br>Johannes-Kepler-Gymnasium, Leonberg, Germany |

## Publications

| | |
|---|---|
| 2012 | **The PGC-1 coactivators promote M2 polarization of tissue macrophages in skeletal muscle.**<br>In preparation<br>**Eisele PS**, Beer M and Handschin C |
| 2012 | **The PGC-1 coactivators repress the transcriptional activity of NF-κB in skeletal muscle cells.**<br>Under review at J Biol Chem.<br>**Eisele PS**, Salatino S, Sobek J, Hottiger MO, Handschin C |
| 2009 | **A high-mobility, low-cost phenotype defines human effector-memory CD8+ T cells.**<br>Blood. 2009 Jan 1;113(1):95-9<br>Zenhaeusern G, Gubser P, **Eisele P**, Gasser O, Steinhuber A, Trampuz A, Handschin C, Luster AD, Hess C |

2008 **Distinct motifs in the chemokine receptor CCR7 regulate signal transduction, receptor trafficking and chemotaxis.**
J Cell Sci. 2008 Aug 15;121(Pt 16):2759-67
Otero C, **Eisele PS**, Schaeuble K, Groettrup M, Legler DF

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